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CANCER RESEARCH

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Different Types of Carcinogens and Their Possible Modes of Action: *A Review**†

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Of the many aspects of cancer research, three which are perhaps of particular interest to chemists are the problems of cell metabolism, of chemotherapy, and of carcinogenesis. All these aspects are closely related, and at the present stage of development of the subject chemotherapy and carcinogenesis are intimately connected, as most of the physical and chemical agents used in therapy of cancer are themselves carcinogenic. The correlation between these two properties has been so close in the past few years that, when a new agent is introduced for therapy, one is immediately suspicious that it may be carcinogenic.

From the human point of view, the two aspects of the cancer problem which are most important are prevention and cure. Experiments on chemotherapy are likely to lead to control of the disease and a study of carcinogenesis to prevention or reduction of the incidence. The investigation of carcinogenesis might reduce the incidence of the disease in at least two ways. One of these is the identification of carcinogens which might be acting as extrinsic carcinogenic agents in the external environment. It is obvious that any known carcinogens should be avoided, but they may be difficult to recognize. One approach to carcinogenesis is through the examination of the incidence of cancer in sections of the population. In the case of chimney-sweeps and the early operators of x-ray apparatus the incidence was so great as to be obvious, but careful statistical analysis might lead to the recognition of many, as yet unsuspected, extrinsic carcinogenic factors.

This method has until now been mainly instrumental in demonstrating carcinogenic factors concerned with occupations such as the bone cancer of girls who painted the luminous dials of watches, the bladder cancer of men working with β-naphthylamine, and the scrotal cancer caused by the lubricating oils used in the spinning mills in the Lancashire cotton industry. The incidence of all these forms of cancer has been greatly reduced by the introduction of precautions based on our knowledge of carcinogenesis. A great deal of cancer not due to special occupational risks could be reduced by application of similar principles. The increase in the incidence of cancer of the lung of man which has occurred during the present century is probably due to increased contact with carcinogenic stimuli. We have reason to be suspicious of the air we breathe, the food we eat, and the medicaments we use, and such suspicions should be allayed or justified by research.

Fundamental knowledge of carcinogenesis might lead to prevention of the disease in another way. The effect of carcinogens may be merely to increase the incidence of the change of normal cells to cancer cells, a change which is almost certainly analogous to a mutation or sport, and may occur to a small extent without any external agent. Understanding of the nature of this change might even lead to its prevention, and so reduce that part of cancer incidence in which no external stimulus was involved. It is of course possible that there would be no cancer without external stimuli, but that would seem to be unlikely.

The recognition of the carcinogenic nature of

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chimney soot and coal tar was followed by the isolation of the pure carcinogenic hydrocarbon 3,4-benzpyrene. This led to the synthesis and testing of a large number of carcinogenic polycyclic hydrocarbons by Cook and Kennaway (18, 19) in England and Fieser and Shear in the United States. Progress in this field was rapid in the thirties, partly because of the effectiveness of the biological tests for substances of this type. The application of the substances by painting on the skin of mice or injection into mice or rats produced easily visible papillomas, epitheliomas, or sarcomas. During the last 10 years consideration of the essential nature of the carcinogenic hydrocarbons had indicated that activity was usually associated with the presence in the molecule of a region of high electron density. Such consideration was made much easier by the publication of the survey of compounds tested for carcinogenic activity by Hartwell (30). Sir Robert Robinson (45) pointed out that carcinogenic hydrocarbons usually contained a phenanthrene double bond, and the French workers, Daudel (21) and Pullman (44), made many theoretical calculations showing that the carcinogenic activity was dependent on a high electron density of a part of the molecule which Schmidt (46) had termed the K region—the K standing for Krebs or Cancer. The conclusions of the theoretical chemists have in general been supported by the determinations of the rate of oxidation by osmium tetroxide made by Badger (1), following the study of this reaction made by Criegee (20). That all carcinogenic hydrocarbons contain a region of high electron density or, in other words, a bond with considerable double bond character in Pauling's sense (43) seems to be established. It has never been claimed that all substances which contain an active double bond or a region of chemical unsaturation are carcinogenic, so that the presence of an active K region is only one requisite of a carcinogenic molecule. On the other hand, there are a number of carcinogenic substances outside the groups of hydrocarbons which have no typical K region.

The activated phenanthrene double bond is only one type of a number of chemical groups which appear to confer carcinogenic activity on molecular structures. The term "carcinogenophore" analogous to chromophore has been suggested (9, 10) as a general term for such groups in carcinogenic molecules and the term auxocarcinogen for groups which enhance the chemical activity and biological activity of such structures. Although many carcinogenic hydrocarbons are known, they form only one group of carcinogens. As long ago as 1938, Haddow (26) drew attention to "the striking multiplicity of tumour-producing agents including the hydrocarbons and radioactive agents." His own work had shown that the carcinogenic hydrocarbons, like the carcinogenic radiations, inhibited the growth of tumors and animals. Haddow suggested that "the carcinogenic agents operate by producing interference with certain normal functions of the cell in such a way as to induce variation." The total number and types of known carcinogens has increased enormously since 1938, and our knowledge of their biological action has also increased; but the similarity of action between the carcinogenic chemicals and the ionizing radiations is known with more certainty and is even more striking.

In describing a substance as carcinogenic, I propose, for the purpose of this discussion, not to differentiate between sites and animal species in which carcinogens sometimes show remarkable specificity. Thus, if an agent produces tumors in any site of any species it is considered a carcinogen. An interesting example of this specificity is sodium arsenite, which seems to be carcinogenic for man (40) but not in any laboratory animals in which it has been tested.

The known carcinogenic agents can be conveniently divided into four groups: physical, inorganic, aliphatic, and aromatic. The physical agents include the application of localized cold in the form of carbon dioxide snow (4), implanted cellophane (41), and the ionizing radiations. Although the ionizing radiations are physical agents, they are thought to produce their biological effects by liberation of free hydroxyl radicals and so have a chemical mechanism. Tumors appear to have been induced by a number of inorganic agents including metallic nickel (33) and by salts of zinc (2) and beryllium, but the difficulties of testing inorganic substances are possibly greater than with the aromatic compounds.

Investigation of aliphatic carcinogens has been largely a post-war development, but a number (cf. Table 1) are now known. During the war it was recognized that the vesicants mustard gas and the nitrogen mustards resembled the ionizing radiations in several biological actions. Compounds which produce some or all the effects of radiations and the effects themselves have been described as radiomimetic (22). Many effects of this type are known, but for the purpose of the present discussion perhaps the most interesting are the inhibition of cell division and of growth, the induction of mutations and of specific chromosome damage. If treatment with an agent produces these effects, then there appears to be some chance that the treatment will also produce cancer, because these

effects are produced by known carcinogens. Because of this correlation, Horning and I (13) tested the nitrogen mustards in mice and found them to produce tumors, although rather slowly. Heston (32) and Haddow have since induced tumors with sulfur mustard or mustard gas. Experiments on the effect of substances of this type on chromosomes of the nuclei of tumors growing in rats and on the inhibition of growth of tumors indicated that only substances with at least two active chemical centers were effective. Thus, methylbis(β-chloroethyl)amine, the usual nitrogen mustard, is active, but dimethyl-β-chloroethylamine is inert (12). Following such observations, some of Haddow's co-workers (25) suggested that these substances acted as cross-linking agents. With this hypothesis in mind, diepoxides including butadiene diepoxide were investigated and found to be radiomimetic agents, and some of these have induced tumors in animals. Other substances of this type which have been found to be carcinogenic are trimethylol melamine (31), which has also been used in the clinical treatment of leukemia (42), and the dimesyl α-ω glycols, which have been investigated by Haddow and Timmis (29) and of which 1,4-dimethyl sulfonoxybutane may be a useful drug for treatment of myeloid leukemia (24). All these compounds are able to esterify acid groups, possibly through the intermediate formation of carbonium ions, and it is probable that these substances produce their effects in the body by reactions of this type.

Induction of tumors is one of the radiomimetic effects which is more difficult to produce than induction of chromosome breakage. Now, although chromosome breaks can be induced with monofunctional compounds such as ethyleneimine (6), bifunctional compounds are almost a hundred times more active in this respect. Interesting monofunctional carcinogenic compounds of this type, however, are the stearoyl ethyleneimine and caproyl ethyleneimine (31).

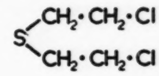
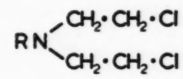
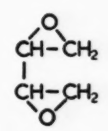
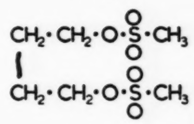
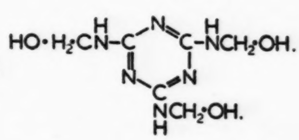
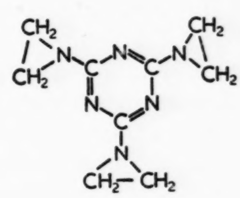
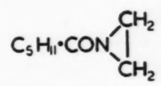
Urethan, or ethyl carbamate, induces lung tumors in mice and is also used in the chemotherapy of leukemia. The specificity of ethyl carbamate is remarkable, as methyl carbamate, propyl, and butyl carbamates are inactive.

The aromatic carcinogens can be divided rather arbitrarily into three classes: the hydrocarbons, the aromatic heterocyclics, and the aromatic amines.

Most of the carcinogenic hydrocarbons are derivatives of phenanthrene or anthracene in which the chemical activity is increased by substituent groups such as methyl or condensed benzene rings which do not destroy the planarity of the molecule (Table 2). Thus, the simplest carcinogenic hydrocarbons are 1,2,3,4-tetramethylphenanthrene, 9,10-dimethyl anthracene and 1,2-benzanthracene, but the parent hydrocarbons, phenanthrene and anthracene, are inactive. In each of these three cases, as in most carcinogenic aromatic hydrocarbons, there are probably at least two centers of specific chemical reactivity—thus, the 9,10 double bond, and the 1,4 positions of the tetramethylphenanthrene, the 9,10 meso positions, and probably the α-β (1,2) bonds of 9,10-dimethylanthracene and the 3,4 bond and 9,10 meso positions of 1,2-benzanthracene are all active centers. The ability to partake in 1,4 addition reactions is possibly important in carcinogenic hydrocarbons; the meso positions which can partake in such reactions may be carcinogenophores. Examined in this light, many carcinogenic hydrocarbons are found to have two carcinogenophores. The carcinogenic

TABLE 1

ALIPHATIC AND HETEROCYCLIC CARCINOGENS.

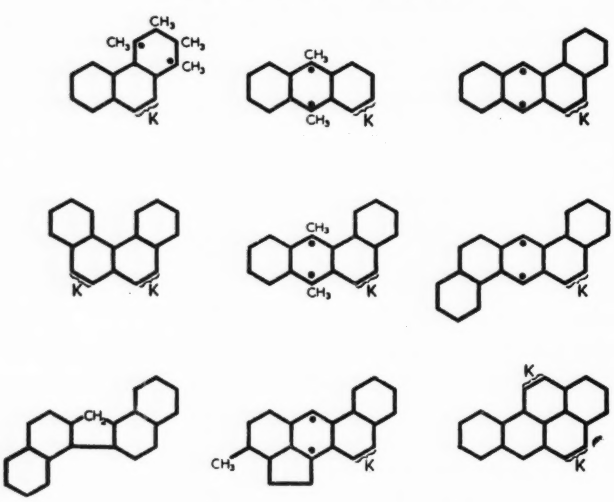
MUSTARD GAS.	
NITROGEN MUSTARDS.	
BUTADIENE DIEPOXIDE.	
DIMESYL GLYCOLS.	
TRIMETHYLOL MELAMINE.	
2:4:6-tris-TRIETHYLENEIMINO-S-TRIAZINE.	
CAPROYL ETHYLENEIMINE.	
URETHANE.	$CH_3 \cdot CH_2 \cdot O \cdot C \cdot NH_2$

1,2,5,6-dibenzanthracene, 3,4-benzphenanthrene, and 3,4-benzpyrene each have two phenanthrene double bonds or K regions. With the dibenzfluorenes it is possible that bonds in each of the naphthalene nuclei are active. Thus, in the case of most carcinogenic hydrocarbons, two carcinogenophores can be indicated, if one accepts the meso positions of anthracene or analogous positions as such.

A large number of aromatic heterocyclic carcinogens are known (Table 3): thus, dibenzcarbazoles (11) and dibenzacridines (3) are carcinogenic, but the introduction of a second heterocyclic nitrogen as in 1,2,5,6-dibenzphenazine destroys activity. French investigators (35) have carried out much work on the benzacridines, showing that the carcinogens of this series have K regions of particularly high electron density. In addition to this, the active methyl benzacridines have the heterocyclic nitrogen atom as a center of unsaturation which is possibly a carcinogenophore.

TABLE 2

CARCINOGENIC HYDROCARBONS.



The 4,7-dimethyl-2,3,5,6-dibenzthionaphthene (5) is an interesting compound in which the sulfur atom replaces the phenanthrene double bond of a carcinogenic 1,2-benzanthracene. As the sulfide linkage can replace the phenanthrene double bond, it too must be considered as a possible carcinogenophore.

Many aromatic amines have been shown to be carcinogenic (Table 4). The simplest of these is β-naphthylamine, which is only known to produce cancer of the bladder in man and in the dog. Because of this particular specificity of site of action, it seems possible that the biological effect may be due to a metabolite of β-naphthylamine excreted in the urine.

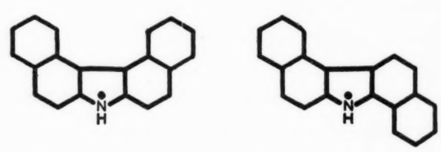
There are a large number of carcinogenic amines with two benzene groups. The first group of compounds of this type to be discovered were the azo compounds—the aminoazotoluenes and the N-methylaminoazobenzenes. An interesting point about these aminoazobenzenes is that at least one methyl substituent group is necessary for carcinogenic activity. Aminoazobenzene is inactive, but N-methylaminoazobenzene and butter yellow (4-N,N-dimethylaminoazobenzene) are active, as is *o*-aminoazotoluene. Thus, the methyl group can be attached to carbon or nitrogen. These essential methyl groups, like those of carcinogenic hydrocarbons, may be merely repelling electrons and so increasing the activity of some other part of the molecule, or they may react directly with some cell constituent.

The carcinogenic aminostilbenes which were discovered by Haddow, Harris, Kon, and Roe (28) have a formal similarity to the azo compounds with the ethylene group replacing the azo group. These tumor-producing aminostilbenes were discovered through their high activity in inhibiting the growth of tumors. In the stilbenes and azo compounds unsaturated groups occur as the amino group, the stilbene double bond and the azo group.

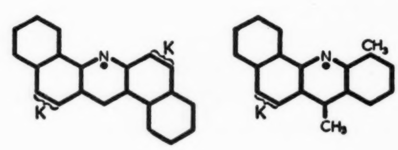
TABLE 3

AROMATIC HETEROCYCLIC CARCINOGENS.

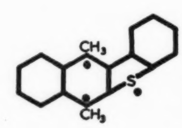
CARBAZOLES



ACRIDINES



4:7 DIMETHYL 2:3:5:6-DIBENZTHIONAPHTHENE.



In the aminoazobenzene and aminostilbene series the introduction of a methyl or chloro group into the 4′ position of the molecule appeared to destroy the carcinogenic or growth-inhibiting effect. If the group introduced is a fluoro group, the activity is increased, and 4′-fluoro-4-dimethylaminoazobenzene (38) appears to be the most potent of the aminoazo compounds.

In experiments on the structural requirements of carcinogens related to acetylaminofluorene,

Miller, Miller, Sandin, and Brown (37) found that dimethylxenylamine (*p*-dimethylaminodiphenyl) was carcinogenic. This molecule is like the carcinogenic azo compounds or stilbenes without the azo or ethylene group. Unless one writes this structure in a quinonoid form, for which there seems to be no real reason, it appears to have only one reactive center.

The aminodiphenyl compound is a link between aminoazobenzene and aminostilbenes and the aminofluorene derivatives. Aminofluorene and acetylaminofluorene are carcinogenic, and Miller and his colleagues (37) have found that a number of analogs of acetylaminofluorene are carcinogenic. These include acetylaminodibenzthiophene, acetylaminodibenzthiophene oxide, and acetylaminodibenzfurane and dimethylaminodiphenyl. In this series consideration of the structures of active compounds suggests that the shape of the part of the molecule attached to the amino group is of great importance but that the atoms forming that shape can vary considerably.

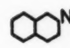
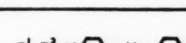
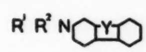
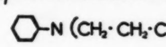
The aromatic nitrogen mustards which were developed by Haddow, Kon, and Ross (see 27) form another group of carcinogenic amines. These are compounds in which the aliphatic residue of the nitrogen mustard molecule is replaced by aromatic residues, such as substituted or unsubstituted phenyl or naphthyl residues. Such compounds are much less toxic than the aliphatic nitrogen mustards, and one of them, the β-naphthyl-bis(β-chloroethyl)amine has been used in the clinical treatment of Hodgkin's disease and has induced tumors in mice and rats. Although it is an aromatic compound, its chemical and biological properties are closer to the aliphatic carcinogens than to the other aromatic amines. Thus, the active chloroethyl groups have a direct and obvious chemical reactivity, since such groups can esterify acid residues or react with amino groups.

The carcinogenophoric groups in typical aromatic compounds include the phenanthrene and stilbene double bonds, the meso positions of anthracene or tetramethyl phenanthrene, the azo group, the hetercyclic nitrogen, the aromatic amino group, and the sulfide link. Now all these groups are capable of reacting with oxidizing agents such as perbenzoic acid and are considered unsaturated groups. With the outstanding exception of the dimethylxenylamine, most of the aromatic carcinogens appear to have two such centers of unsaturation. Whether two such groups are really essential and, if so, what part they play in the biological actions are still matters for conjecture and experiment.

This brief survey of the different types of carcinogenic agents indicates the wide variety of causes of cancer, although the effects are, to some extent, specific for each type. This diversity of structure probably means that the same end can be brought about by a variety of means. The author made the suggestion (8) that the carcinogens produced their effects by inhibition of enzymes such as the phosphokinases. Investigation of this hypothesis has not been very fruitful, thus the reduction in glycolysis of tumor tissue of animals treated with nitrogen mustard occurred after the chromosome damage (12). This is therefore more likely to be an effect of cell damage rather than the cause of chromosome changes. The reduction of glycolysis of tissues receiving moderate doses of x-rays is also negligible. Although some carcinogenic compounds may produce their specific effects by enzyme inhibition, the hypothesis that the results are due to the more direct action on chromosome nucleic acid seems more likely in some cases. Because the carcinogens produce specific damage to chromosomes and chromosomes contain desoxyribosenucleic acid, it is tempting to assume that the carcinogens act by producing abnormalities in the nucleoprotein. Such abnormalities might be induced in different ways.

TABLE 4

CARCINOGENIC AROMATIC AMINES.

I	 β-NAPHTHYLAMINE
II	 ACTIVE WHEN R^1 *or* R^2 = CH_3 *and* X *is* –N=N– *as in* AMINOAZOBENZENE X *is* –CH=CH– *as in* AMINOSTILBENE *or* X *is a single bond as in* AMINODIPHENYL
III	 *active when* Y *is absent as in* AMINODIPHENYL. *is* CH_2 *as in* AMINOFLUORENE. *is* –O– *as in* AMINODIBENZFURANE. *is* –S– *as in* AMINODIBENZTHIOPHENE. *is* –SO– *as in* AMINODIBENZTHIOPHENE OXIDE.
IV	AROMATIC Bis (β-HALOGENOETHYL) AMINES eg: 

The most direct way in which damage in the nucleic acid might produce the observed effect in the chromosomes is by cleavage of the nucleic acid chains. Such fission occurs when solutions of desoxyribosenucleic acid are exposed to x-rays (47) or treated with mustards (17). This has been fol-

lowed by measuring the change in viscosity and also by the decrease in molecular weight as measured in the ultracentrifuge. Thus, x-rays, which probably act by producing free hydroxyl radicals *in situ*, and the mustards may produce their carcinogenic and mutagenic effects by directly destroying chromosome nucleic acid.

Other substances chemically allied to the mustards, such as butadiene diepoxide, do not cause fission of nucleic acid *in vitro* but are effective cross-linking agents. In the case of such substances it is possibly best to assume, as a working hypothesis, that they produce abnormalities and genetic changes by producing cross linkages in the nucleoprotein of the chromosomes.

Other carcinogens, such as beryllium, precipitate nucleic acid and may be functioning in this way. Such precipitation may be a form of cross linkage by salt formation with a divalent ion. It has been suggested that the effects of beryllium are due to inhibition of cellular phosphatase, although the data on such inhibition (34) would not indicate that this is a likely mechanism.

The biochemical mechanism underlying the action of aromatic carcinogens is still not known, but two lines of investigation may throw light on the subject. The first investigations are concerned with reaction of aromatic compounds with purines and pyrimidines. Weil-Malherbe (48) and Neish (39) had shown that aromatic carcinogens dissolve in solutions of purines such as caffeine and tetramethyluric acid to form complexes. Dr. J. Booth and I have investigated this reaction further and have found that aqueous solutions of nucleic acid as well as simpler purines dissolve polycyclic hydrocarbons and aromatic amines. The amount of hydrocarbon which dissolves increases with increase in concentration of purine and depends upon the nature of the purine. Although these complexes are easily broken down, the results show that the aromatic compounds including carcinogens do combine—if only loosely—with nucleic acid and that this association may change the nucleic acid sufficiently for the chromosome aberrations to result. The fact that caffeine combines more readily with benzpyrene than does nucleic acid means that the caffeine would compete with the nucleic acid for the hydrocarbon and so reduce the effect on the chromosomes. This would account for the observation that the carcinogenicity of benzpyrene was reduced when it was injected in tetramethyluric acid solution (49). Thus, simple purines are possibly anti-carcinogenic agents.

The study of the metabolism of carcinogenic and noncarcinogenic aromatic compounds has now been pursued for many years. The noncarcinogenic hydrocarbons, naphthalene (50, 7), anthracene (14), and phenanthrene (16) are converted to phenols and dihydroxy dihydro derivatives or "diols" in the body. As the diols are easily dehydrated to phenols by treatment with acid, it was thought that the diols were the biological precursors of the phenols. During the last year, however, we have found (15) that the processes of phenol formation and diol formation are separate and independent. If rats are injected with naphthalene for the first time, they convert some of the hydrocarbon into α-naphthol, but after repeated injection they convert increasing amounts of the hydrocarbon into the diol. At the same time that the animals are trained by injection of the hydrocarbon the lethal dose increases, giving clear evidence of adaptation or drug resistance. There are two independent metabolic pathways for naphthalene, and the same alternatives may be possible for more complex carcinogenic compounds. As the metabolism of the hydrocarbons seems to be associated with carcinogenesis, one of these processes, or both, may be the cause of chromosome damage and cancer development. If the oxidative process occurs while the hydrocarbon is held in the nucleic acid of the cell, then the nucleic acid might also be modified at the same time. If the oxidation were of the type leading directly to a phenol, it is likely to involve free radicals, and the free radicals produced *in situ* would be expected to disrupt the nucleic acid. If the oxidation were analogous to the production of the dihydroxydihydro derivative, then an epoxide is a very probable intermediate product. Such an epoxy derivative might then act like the aliphatic carcinogenic epoxides.

For the carcinogenic action of urethan, still another mechanism must be postulated. Urethan is relatively inert in so far as very large doses must be given to produce any biological action and the action is produced only slowly. In order to produce the same degree of chromosome damage, 10,000 times as much urethan must be used as nitrogen mustard, and the effect takes 24 hours longer to be produced. McKinney (36) showed that urethan is much more effective than methyl carbamate as an inhibitor of the transmethylation of glycocyamine or nicotinamide by methionine in tissue homogenates. An analogous inhibition of methylation of uracil might lead to deficiency of thymine, an essential constituent of the desoxyribosenucleic acid. In experiments carried out with Dr. Koller, partial neutralization of the chromosome-damaging action of urethan was observed when thymine was administered with the urethan.

There are thus at least five hypothetical processes (see Table 5) by which the changes in nucleic

acid which might lead to cancer can be brought about. Of these the process involving fission of the nucleic acid by free radicals produced by radiation or other means or by mustards seems to be the least uncertain. In this a change resembling that seen in the cell can be seen to take place in the nucleic acid treated *in vitro*. Of the other suggested three processes, precipitation by salt formation, cross linking by known cross-linking agents, and complex formation with hydrocarbons may be related in that the nucleic acid reacts with a divalent or bifunctional reagent. The mechanism suggested for the action of urethan would be similar to the effect of choline deficiency in causing cancer in animals (23). By choline deficiency a shortage of

TABLE 5

Carcinogen		Possible action on desoxyribonucleic acid
Group	Type	
Physical	Cellophane	
	Local cold	
	X-rays	Depolymerize
Inorganic	Ni, Na_2HAsO_3	
	$BeCl_2$, $ZnCl_2$	Precipitate
Aliphatic	Mustards	Depolymerize
	Diepoxides	Cross link
	Ethyleneimines	Cross link
	Dimesyl glycols	Cross link
	Urethan	Inhibit synthesis
Aromatic	Hydrocarbons	Form complex
	Heterocyclics	Form complex
	Amines	Form complex

methyl donors would have a similar final effect as inhibition of the means of using such methyl donors as choline and methionine.

It is clear that there is much work to be done by chemists, biochemists, pharmacologists, and pathologists before we know enough about the processes involved to be able to prevent the occurrence of cancer even from external carcinogens. It should eventually be possible to reduce the incidence of human cancer by application of our knowledge of the nature of carcinogens and the processes by which they operate.

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Alkaline Phosphatase Activity in Epithelial Metaplasia*

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The etiology of epithelial metaplasia and its relation to neoplasia have been of interest for many years. Investigators have proposed (39, 41) and opposed (6) the view that metaplastic transformations in various sites may be precancerous. It seems well established that such alterations often accompany neoplastic growths (20, 25, 27, 32). The need to distinguish metaplastic from neoplastic lesions in the genital tract has received emphasis in recent pathologic studies (7, 33, 34, 35, 43).

McCullough and Dalldorf (31) have suggested that all squamous metaplasia and keratinization result from the same etiologic factor, local vitamin A deficiency, regardless of the initiating stimulus (e.g., mechanical, hormonal). Investigations on the effects of vitamin A on estrogen-induced vaginal cornification (19, 23, 47) and on epidermal differentiation (44, 45) have given some support to this suggestion. Earlier studies suggested that metaplastic lesions in the male rat genital tract caused by estrogen treatment and those accompanying avitaminosis-A are histologically identical (21). Such histologic identity, however, does not hold for all types of genital epithelial metaplasia in the male rabbit (9). In the male mouse, it has been shown that vitamin A deficiency intensifies the histologic response in the genital tract to minimal dosage with estrogen (15).

In the course of a series of studies on the relation of steroids and phosphatases in the male genital tract (11, 12), it was noted that estrogen-induced epithelial metaplasia is accompanied by alkaline phosphatase activity in the replacing epithelium. This phenomenon has been investigated further in both estrogen-induced and avitaminotic-A lesions, in addition to other instances of metaplasia; results are reported herein. Differences in the histologic picture of the metaplastic process after estrogen treatment and in vitamin A deficiency were seen in (*a*) the mode of growth of the lesion, (*b*) the alkaline phosphatase activity of the replacing epithelium, and (*c*) the extent of involvement of the various reproductive structures.

MATERIALS AND METHODS

Tissues with estrogen-induced metaplastic lesions were obtained from estradiol-treated male mice, rats, guinea pigs, and rabbits used in previous studies (11, 12). All sex accessory and duct tissues removed at the time of the termination of these experiments were examined for evidence of epithelial metaplasia.

A total of 21 male mice and 13 male Long-Evans rats was used in the study of avitaminotic-A lesions. Of these, 8 mice and 10 rats provided genital tissues with distinct evidences of epithelial metaplasia. Portions of submaxillary salivary glands and trachea were occasionally removed to check on the efficacy of the vitamin A-free diet. Both mice and rats were maintained on a standard vitamin A test diet (18).

Other instances of epithelial metaplasia observed in previous studies are also considered: metaplastic transformations accompanying methylcholanthrene-induced squamous-cell carcinomas of the rat ventral prostate (12) and certain spontaneous lesions of the male rabbit genital tract (9).

Alkaline phosphatase activity was demonstrated histochemically by the Barger (8) modification of the Gomori technic. Acetone-fixed tissue sections were incubated at 37° C. for 18 hours in sodium glycerophosphate at pH 9.5. Control slides were processed identically, except for poisoning of the substrate with approximately 0.01 M KCN. It is of interest that certain batches of control slides incubated for 18 hours in poisoned substrates wherein the KCN concentration was low showed some evidence of alkaline phosphatase activity in the metaplastic epithelium, although other intensely active areas were completely negative (cf. 16). Nucleic acids were demonstrated in some sections with pyronin-methyl green (Pappenheim-Saathof).

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OBSERVATIONS

Estrogen-treated mice.—Estrogen-induced metaplastic changes in the male mouse, first described by Lacassagne and Villela (26) and Burrows (13), and well reviewed by Thorborg (47), have been discussed in detail previously (11, note Figures 5–10). In brief, a proliferation of alkaline phosphatase-positive basal cells has been observed in the anterior prostate, dorsal prostate, and seminal vesicle. This proliferation is most extensive in the anterior prostate and culminates therein in the transformation of many of the alveoli into small keratinized nodules (Fig. 5). Eventually, the original epithelium, which does not show alkaline phosphatase activity, is entirely sloughed, and the alveolar lumen partially or wholly obliterated by keratin. As noted by Burrows (13), the process in the dorsal prostate does not progress to keratinization. No metaplastic alterations were seen in the ventral prostate, the epithelium of which is normally alkaline phosphatase-positive. This gland becomes quite atrophic after castration, with or without estrogen treatment, and upon estrogen treatment of intact mice.

Avitaminotic-A mice.—It is ordinarily very difficult to produce the syndrome of avitaminosis-A in adult mice, inasmuch as the minimal daily requirement of vitamin A is only 1 unit (30). However, we have found that placing mice on vitamin A-free diet immediately after weaning occasionally results in the development of severe symptoms of vitamin A deficiency in the male genital tract within 2 months or less. It has been pointed out that the ability to produce the syndrome in mice is dependent upon "the stringency of prenatal and lactational treatment" (30, p. 382).

Despite the occurrence of an actively keratinizing, stratified squamous replacing epithelium, the histologic picture in sex accessories of male mice with vitamin A deficiency suggests some real differences from that found after estrogen treatment. Proliferation of alkaline phosphatase-positive basal cells was not seen in any of the avitaminotic-A accessories. The occurrence of multiple foci of basal cell proliferation, which keratinize and spread underneath the original epithelium, as described in the rat (49), was not noted. Instead, our findings agree with the general condition described previously in the mouse of "an orderly layer of keratinizing cells which seemed to begin at only one point and grow in all directions" (51, pp. 187, 189). Growth seemed to occur in part, at least, over the surface of the original epithelium, rather than underneath.

The metaplastic changes first occur in the luminal epithelium of a structure such as the seminal vesicle, the glands being initially unaffected (Fig. 1). In general, once having begun, the metaplastic growth seems largely to extend "centrifugally" (i.e., away from the lumen), instead of "centripetally" (i.e., toward the lumen) as occurs after estrogen treatment. In contrast to the situation occurring after estrogen administration, wherein only the anterior prostate is transformed into a keratinized mass, all the structures with avitaminotic-A lesions (seminal vesicle, ductus deferens, anterior and dorsal prostates, epididymis) show the same picture (Figs. 1–2). However, as after estrogen treatment, the anterior prostate is most readily and most noticeably affected. In their earlier study, Wolfe and Salter (51) did not find changes in the epididymis.

Intra-epithelial keratinization can occasionally be seen, and this may represent the "multiple foci" of Wolbach and Howe (49). As keratinization proceeds and involves the entire epithelium, the intra-epithelial "pearls" seem to be largely sloughed into the lumen. The cells involved are alkaline phosphatase-negative (Fig. 4). Even in the anterior prostate, where the metaplastic process eventually results in keratinized alveoli structurally indistinguishable from those resulting from estrogen treatment, such epithelial lesions are entirely alkaline phosphatase-negative (Fig. 6).

Estrogen-treated rats.—No evidence of genital tract keratinization was found in rats castrated and treated with estrogen for as long as $5\frac{1}{2}$ months. However, alterations interpreted as metaplastic were noted in rats so treated for 4 and $5\frac{1}{2}$ months. As in the estrogen-treated mouse, these changes consisted of groups of alkaline phosphatase-positive basal cells, which were seen in the ductus deferens (Fig. 8), seminal vesicle (Figs 7 and 9), and the ducts of the dorsal prostate. These aggregations may become quite extensive and result in the transformation of the glands of the seminal vesicle into adenoma-like growths (Fig. 9). The anterior prostate, so reactive in the mouse to estrogen, was found consistently unaffected in the rat. Again, no metaplastic alterations were seen in the ventral prostate.

Avitaminotic-A rats.—The avitaminotic-A lesions occurring in the genital tract are similar to those of the mouse, except that the superficial layers (strata granulosum and corneum) often show a coloration indicative of alkaline phosphatase activity, although the more basal cell layers are always negative (Fig. 10). This "activity"—in dead and dying cells—may be an artifact. No proliferation of alkaline phosphatase-positive basal cells was seen. The original description of

the process (49) was difficult to verify, at least as concerns "the multiple origin of keratinizing foci." Although the keratinizing process may originate as subepithelial centers which break through the overlying original epithelium, it does not seem that proliferation occurs consistently underneath the original epithelium. The possibility of superficial spreading of keratinization is certainly suggested (Fig. 10).

Lesions were observed in the seminal vesicle, anterior and ventral prostates, and preputial gland. In ventral prostate tissue affected by the deficiency in vitamin A, premetaplastic atrophy and pluristratification were accompanied by a loss of the epithelial alkaline phosphatase normally characteristic of this organ (Fig. 11). Keratinizing epithelium again was alkaline phosphatase-negative in its basal layers. The anterior prostate was relatively unresponsive, as compared to the extensive response of the homologous structure in the mouse.

Estrogen-treated guinea pigs.—A distinct single layer of basal cells is evident in the seminal vesicle and ductus deferens of normal intact and of untreated castrate guinea pigs. In the normal animal, this layer is often alkaline phosphatase-active; in the castrate, it is often inactive. In the estrogen-treated castrate, the layer is phosphatase-active and stands out in distinct contrast to the phosphatase-inactive original epithelium (Fig. 13). Metaplasia evidently begins as a result of proliferation of this basal cell layer which breaks through the overlying epithelium to form islands and papilloma-like growths of alkaline phosphatase-positive polyhedral cells (Fig. 13).

Cornification and precornification were observed in the prostate of the guinea pig after estrogen treatment. The alkaline phosphatase-positive metaplastic epithelium is in contrast to the inactive original epithelium. This is well illustrated by the section of a duct of the median prostate shown in Figure 14. Here, one side of the duct is alkaline phosphatase-negative (more or less normal epithelium), and the other side is alkaline phosphatase-positive (stratified squamous epithelium beginning to cornify). The keratinizing prostatic epithelioma first described by Lipschütz *et al.* (28) is alkaline phosphatase-positive.

Estrogen-treated rabbits.—Estrogen-induced metaplasia has been described in detail in the male rabbit urogenital system (10, 14). That it differs from that seen in many other mammals has been recently emphasized (9). Instead of the usual squamous metaplasia often accompanied by keratinization, there is metaplasia to a stratified columnar or polyhedral epithelium, noncornifying, which may completely occlude the alveolar lumina in the prostate and vesicular gland (Fig. 16). It has been termed transitionoid metaplasia (9, 10) because of the resemblance of the replacing epithelium to that normally lining the bladder.

As in other species, the metaplastic epithelium following estrogen treatment of the rabbit is characterized by intense alkaline phosphatase activity. Foci of presumably premetaplastic epithelium can be detected in alveolar walls. The patches spread out and cover the original epithelium in nonmetaplastic areas, in many instances eventually filling the entire alveolus (Fig. 16). The details of the process can be discerned because of the alkaline phosphatase activity of the replacing epithelium. Evidences of nonmetaplastic original epithelium can be seen surrounding the metaplastic masses (Fig. 16). Sometimes bridges of metaplastic cells can be seen partially occluding the lumen, as in the ducts of the bulbourethral gland. In the seminal vesicle, the metaplastic epithelium evidently proliferates as basal cells, which form a distinct alkaline phosphatase-positive layer beneath the original epithelium (Fig. 15).

Spontaneous lesions in the rabbit.—Certain spontaneous metaplastic epithelial lesions have been described in the prostate and vesicular gland of the male Dutch rabbit (9). It was originally thought that these keratinizing lesions might be due to a local inadequacy of vitamin A. However, administration of cod liver oil failed to result in any evidence of their alleviation (9). In addition, we find that this metaplastic formation is alkaline phosphatase-positive (Fig. 3), unlike the avitaminotic-A lesions in the mouse and rat. The etiology of these lesions in the rabbit remains unknown.

Methylcholanthrene-treated rats.—The loss of specific alkaline phosphatase activity accompanying the neoplastic transformation in the ventral prostate of the rat has been discussed elsewhere (12). The parenchyma of squamous-cell carcinomas induced by intraprostatic injection of methylcholanthrene is almost entirely negative for alkaline phosphatase activity, despite the activity of the tissue of origin. In some methylcholanthrene-induced abnormal growths, metaplastic areas can be found (cf. 32) which are often alkaline phosphatase-positive. However, apparently neoplastic downgrowths into the stroma arising from these areas are alkaline phosphatase-negative (Fig. 12).

DISCUSSION

Observations on the rat and mouse suggest that genital epithelial metaplasia caused by estro-

gen administration can be distinguished from that caused by avitaminosis-A on the basis of two principal criteria: (*a*) the consistent "centripetal" growth of replacing tissue in the former, as opposed to the secondarily "centrifugal" growth in the latter, and (*b*) the consistent alkaline phosphatase activity in the former. Basal cell proliferation, leading to pluristratification, occurs in both syndromes. However, the basal cells are alkaline phosphatase-active only after estrogen treatment.

A third difference may lie in the extent of involvement of the several accessories. Thus, the mouse anterior prostate is the principal accessory affected by estrogen treatment, whereas vitamin A deficiency results in cornifying metaplasia in most of the accessories and in the ductus deferens and epididymis. Moreover, in the rat, no keratinization was observed, even after $5\frac{1}{2}$ months treatment with estrogen, although keratinization is extensive in vitamin A deficiency. However, it is of interest that the anterior prostate of the mouse, which is most reactive to estrogen, is also most reactive to a lack of vitamin A.

The alkaline phosphatase activity, as previously mentioned (11), serves almost as a differential stain in the detection of estrogen-induced metaplasia. The details of the process have been described in the mouse without the use of this technic (26, 29, 47); however, the technic may be of some value in distinguishing some metaplastic alterations from histologically similar neoplastic alterations (12). It would be interesting to determine if the metaplastic epithelial alterations seen in association with infarction of the human prostate and after estrogen therapy (7, 33, 35, 43) are alkaline phosphatase-positive, in possible contrast to normal and neoplastic tissue.

In a recent discussion of "epidermization" of the human uterine cervix, Motyloff (34) distinguished between the alteration of normal epithelial characteristics resulting from transformation of the original epithelium (metaplasia) and that resulting from basal cell proliferation (heteroplasia). Adami and McRae's (1) oft-quoted definition of metaplasia states that the process is not direct but involves proliferation from preliminarily dedifferentiated cells or from undifferentiated basal cells. In the male genital tract, alterations resulting from vitamin A deficiency in the rat and mouse appear to include the covering (or invasion) of the original epithelium by stratified squamous epithelium which had proliferated from relatively distant foci. If sheets of proliferating basal cells were continuous under the original epithelium, one would expect the glandular, as well as the luminal, epithelium to be undermined. This does not seem to be the case in the seminal vesicle of rats and mice with vitamin A deficiency (Figs. 1, 10). Coalescence of keratinizing areas in the surface epithelium would eventually obliterate the unaffected glands by "centrifugal" growth. It is difficult to eliminate the possibility of direct transformation of some of the original epithelium. In accordance with Motyloff's terminology (34), local transformation and keratinization would be true metaplasia. According to Adami and McRae (1), direct transformation does not occur in metaplasia, whereas estrogen-induced proliferations from basal cells would satisfy their definition. These latter changes would be termed "heteroplastic" after Motyloff (34).

In view of the evidence for the origin of metaplastic tissue from basal cells *after estrogen treatment*, it is difficult to reconcile some of our observations with Zuckerman's suggestion (52, 53) that estrogen may induce squamous metaplasia only in those tissues "in whose development oestrogen-sensitive [urogenital] sinus epithelium has either played a direct or indirect part" (52, p. 264). The Zuckerman hypothesis has received support in particular from the detailed studies of Raynaud (e.g., 42, pp. 53-54) and, in part, from those of Thorborg (47). It is not proposed to include a detailed discussion of this attractive hypothesis, although some re-evaluation seems warranted. A few points, however, should be made. Along with Thorborg (47), we feel that the distinction between "glandular" and "squamous" response may be invalid. The response of male rabbit sex accessories to estrogen (10, 14) is certainly not squamous regardless of embryonic origin of accessories studied; however, further studies of the embryology of the male rabbit reproductive system are needed.

In any detailed reconsideration of the hypothesis, a careful analysis of the total picture of genital epithelial changes after estrogen will be needed. Thus, our observations and those of other workers on various species of mammals would call for alterations of Thorborg's summary diagram (47, p. 169), at least as it relates to the rabbit, mouse, rat, and guinea pig. In addition, the process of true metaplasia must be defined. Is basal cell proliferation after estrogen treatment (Motyloff's heteroplasia) to be considered as a metaplastic response, regardless of whether or not it ever leads to total epithelial replacement and keratinization?

One point with real bearing on the Zuckerman hypothesis concerns the origin of the basal cells in estrogen-induced metaplasia. Recent work

(3, 4) has indicated that some epidermal cells originate from invading lymphocytes. It was originally believed that this type of cell transformation—epithelium differentiating from a mesodermal lymphoid element—did not occur (1). Possibly somewhat similar is the transformation of macrophages in Triturus into epidermal melanophores which form an integral part of the epidermis (36). The aggregations of alkaline phosphatase-positive basal cells under the original epithelium which occur after estrogen treatment are in close association with the stroma. Since some recent work has questioned the reality of basement membranes (2), one wonders about the possibility of the participation of stromal elements in estrogen-induced metaplastic transformations. On a somewhat different basis, Wells (48) in 1936 indicated that connective tissue cells may transform into epithelium in the metaplastic process in estrogen-treated male ground squirrels. This unorthodox possibility, suggested by histologic observations, seems worthy of further investigation. If verified, it would be difficult to reconcile with the Zuckerman hypothesis. In the guinea pig, a basal cell layer which seems to be a definite lower layer of the epithelium is normally present in certain accessories.

The significance of alkaline phosphatase activity associated with estrogen-induced metaplasia is not clear. The absence of such activity in avitaminotic-A lesions may be related to testicular deficiency and the absence of a substituting steroid. Suggestions as to the role of alkaline phosphatase in fibrous protein (including keratin) synthesis (e.g., 22) have been discussed elsewhere (12) and do not provide a consistent explanation. The basal cell proliferation seen in the estrogen-treated rat does not necessarily result in keratinization. The transitionoid metaplasia occurring in the rabbit (10) never results in cornification. In both cases, the metaplastic cells are alkaline phosphatase-positive. On the other hand, intensely active keratin synthesis in squamous-cell carcinomas of the rat ventral prostate is seldom accompanied by alkaline phosphatase activity, despite the activity of the tissue of origin (12).

The alkaline phosphatase activity may merely be an indicator of cytoplasmic metabolic activity accompanying cellular proliferation (37, 40, 46). Much work exists, however, which points to the relative absence of such activity in many neoplastic growths, even when the tissue of origin shows considerable activity (5, 12, 24). Distinctive pyronin-positive ribonucleic acid concentrations were not seen in the cells showing high phosphatase activity. Differentiation of basal cells, so nicely accomplished with the Barger-Gomori (8) technic, was not observed with pyronin-methyl green. In Lacassagne and Villela's original description (26) of estrogen-induced metaplasia in the mouse anterior prostate, a silver stain was employed. The cytoplasmic fibrils seen with this stain paralleled our observations with the Barger-Gomori technic (11) and allowed a differentiation of the early basal cells. Alkaline phosphatase activity associated with fibril production would be expected if Jeener's suggestion (22) in regard to fibrous protein synthesis were universally applicable.

The validity of the histochemical technic for alkaline phosphatase demonstration has been questioned, particularly with reference to accurate localization of the enzyme (e.g., 38). It is pertinent that with biochemical methods (17) rapidly proliferating (metaplastic?) epithelial growths in the guinea pig uterus after estrogen treatment show high alkaline phosphatase activity. Regardless of what the cytochemical technic used herein may actually demonstrate, and there is evidence that it is alkaline phosphatase which is demonstrated (12), it does allow distinction of metaplastic from original epithelium after estrogen treatment.

In 1933, Wolbach and Howe pointed out that "careful cytological studies [of epithelial metaplasia in vitamin A deficiency] as the deficiency progresses and during the recovery phenomenon may yield interesting correlations between morphology and function" (49, p. 524). One can add to this statement today the desirability of continued biochemical studies involving the possible relationship between vitamin A and estrogen in the processes of metaplasia and keratin formation. On the basis of work reported herein, it seems that metaplastic processes in the male genital tract in vitamin A deficiency and after estrogen administration are not completely identical. Further studies of the development of and recovery from alterations occurring after simultaneous estrogen treatment and vitamin A deficiency seem to be warranted.

SUMMARY AND CONCLUSIONS

1. A study has been made of epithelial transformations in the male genital tract of estrogen-treated mice, rats, guinea pigs, and rabbits; of avitaminotic-A mice and rats; of methylcholanthrene-treated rats; and of rabbits with spontaneous metaplastic lesions.

2. Comparison of metaplastic alterations in mice and rats after estrogen treatment and with vitamin A deficiency reveals three possible histologic distinctions: (*a*) consistently "centripetal"

growth of replacing epithelium in the former compared with secondarily "centrifugal" growth in the latter; (*b*) the occurrence of proliferation of alkaline phosphatase-positive basal cells in the former; and (*c*) the considerably more extensive occurrence of keratinizing stratified squamous epithelium in the latter. Histologic similarity of the metaplastic transformations in the two syndromes may be only superficial.

3. The possible utility of the Barger-Gomori histochemical technic for alkaline phosphatase in distinguishing estrogen-induced metaplasia from neoplastic transformations is suggested.

4. The role of alkaline phosphatase activity in estrogen-induced metaplasia is considered. Possibly it is an indicator of cytoplasmic metabolic activity accompanying non-neoplastic cell proliferation. A constant relation between such activity and keratin synthesis is not supported.

5. The mechanisms of metaplastic transformations require further investigation, with emphasis on the possible relation of vitamin A and estrogen in such changes, and on the origin of replacing epithelium. The Zuckerman hypothesis offering an embryologic explanation for changes observed after estrogen treatment and definitions of metaplasia are briefly considered.

ACKNOWLEDGMENTS

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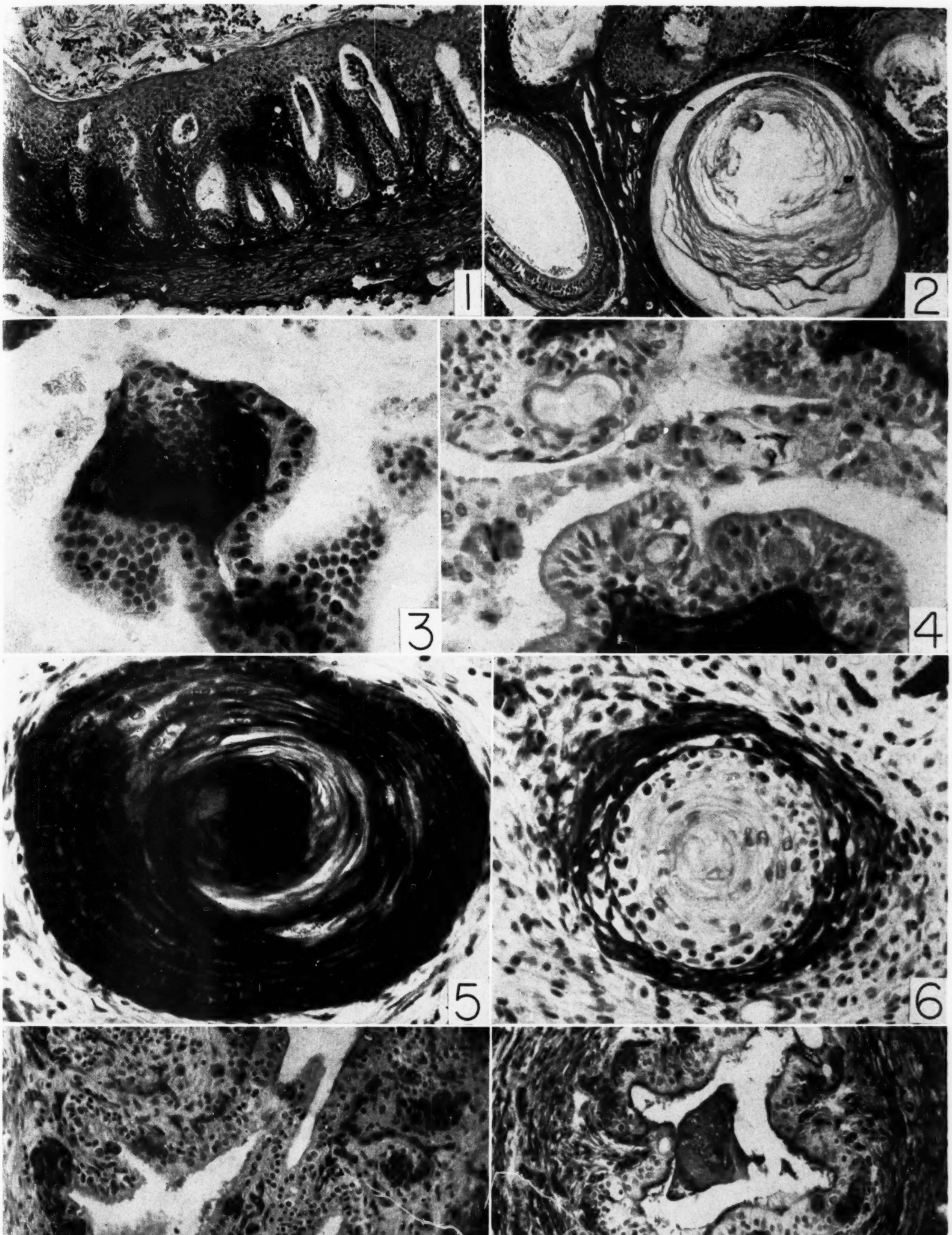
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All photomicrographs are of 6-μ sections of acetone-fixed tissues, treated by the Barger-Gomori technic and lightly stained with hematoxylin. Dense extranuclear coloration is evidence of alkaline phosphatase activity.

FIG. 1.—Seminal vesicle from $7\frac{1}{2}$-month-old male mouse, on vitamin A-free diet for $6\frac{1}{2}$ months. Squamous metaplasia and keratinization of luminal epithelium are unaccompanied by alkaline phosphatase activity. Note relatively unaffected glands, absence of alkaline phosphatase-positive basal cell proliferation, and normal enzyme distribution in fibromuscular wall. $\times 80$.

FIG. 2.—Epididymis of $7\frac{1}{2}$-month-old mouse, on vitamin A-free diet for $6\frac{1}{2}$ months. Portions of duct have undergone squamous metaplasia and keratinization, unaccompanied by alkaline phosphatase activity. Note activity of intertubular stroma. $\times 65$.

FIG. 3.—Spontaneous lesion from vesicular gland of adult intact rabbit. Note alkaline phosphatase-positive metaplastic epithelium. $\times 270$.

FIG. 4.—Portion of anterior prostate alveolus from 5-month-old mouse on vitamin A-free diet for 4 months. Note intra-epithelial "pearl" formation, unaccompanied by alkaline phosphatase activity, positive reaction in circumalveolar stroma. $\times 300$.

FIG. 5.—Anterior prostate alveolus from 6-month-old mouse, castrated and treated with estrogen for 2 months. Note alkaline phosphatase activity accompanying keratinizing squamous metaplasia. $\times 270$.

FIG. 6.—Anterior prostate alveolus from 5-month-old mouse, on vitamin A-free diet for 4 months. Note absence of alkaline phosphatase activity in this squamous metaplastic lesion; stroma retains some activity (cf. Fig. 5). $\times 320$.

FIG. 7.—Seminal vesicle of 7-month-old rat, castrated and treated with estrogen for 4 months. Note proliferation of alkaline phosphatase-active basal cells, inactive original epithelium. $\times 165$.

FIG. 8.—Ductus deferens of 7-month-old rat, castrated and treated with estrogen for 4 months. Note proliferation of alkaline phosphatase-active basal cells, relatively inactive original epithelium (stereocilia show some coloration). $\times 165$.

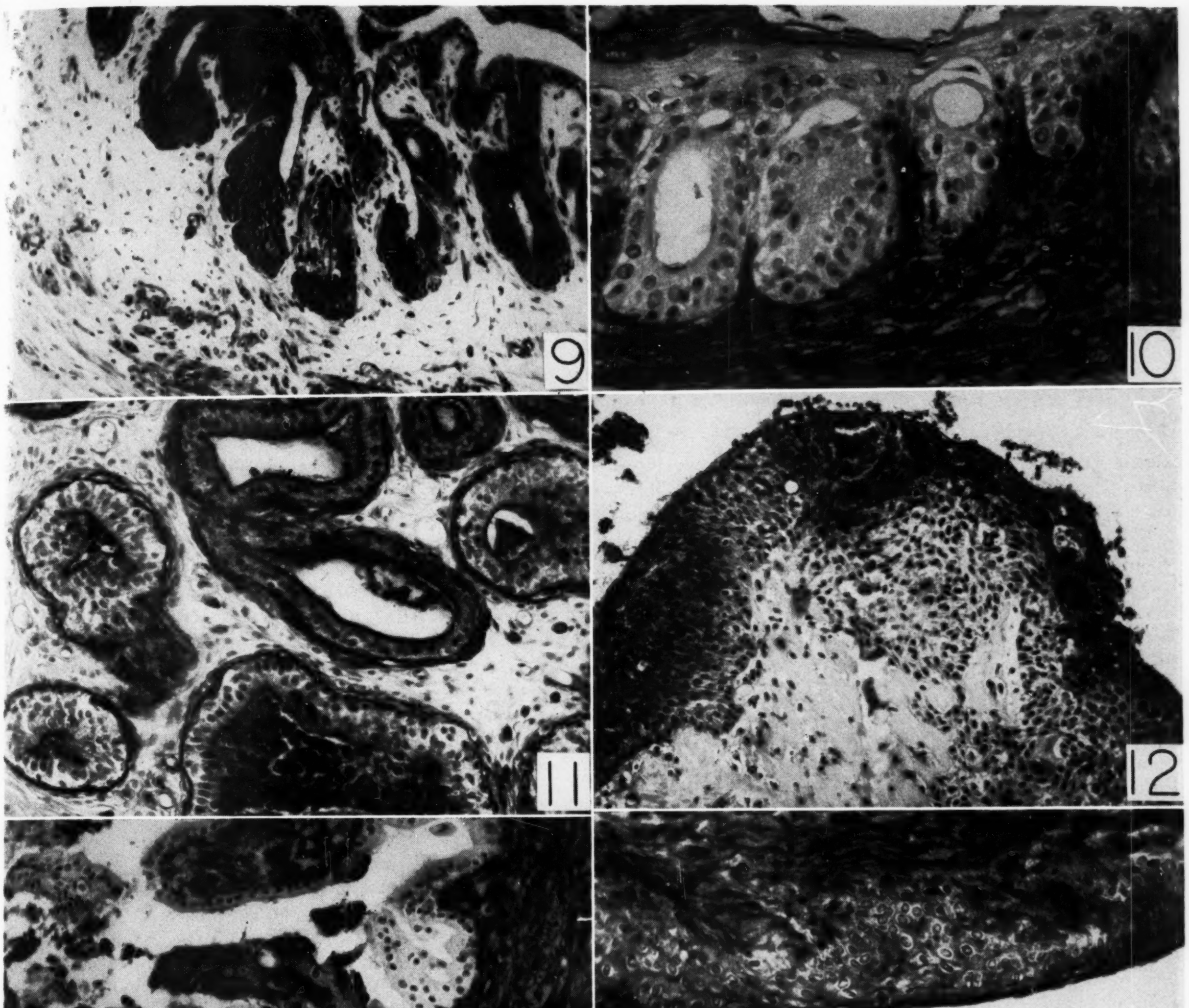
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FIG. 9.—Seminal vesicle of 12-month-old rat, castrated and treated with estrogen for 5½ months. Note transformation of glands into alkaline phosphatase-active adenoma-like structures, a continuation of the metaplastic process seen in Figure 7. ×125.

FIG. 10.—Seminal vesicle of 3¼-month-old rat, on vitamin A-free diet for 2 months. Note keratinizing squamous metaplasia of luminal epithelium, coloration only in some sloughing keratin and in stroma. Glands are relatively unaffected (cf. Figs. 1, 7, and 9). ×300.

FIG. 11.—Ventral prostate alveoli of 2½-month-old rat, on vitamin A-free diet for 7 weeks. Note pluristratification preceding metaplasia with partial loss of normal epithelial alkaline phosphatase activity in some alveoli. ×190.

FIG. 12.—Metaplastic region from ventral prostate of 14-month-old rat after exposure to intraprostatic methylcholanthrene for 7 months. Most of tissue was squamous-cell carcinoma. This region shows alkaline phosphatase-active metaplastic epithelium; cells infiltrating into stroma are inactive. ×140.

FIG. 13.—Seminal vesicle of 10-month-old guinea pig, castrated and treated with estrogen for 3½ months. Note alkaline phosphatase-active basal cell layer, evidently proliferating and breaking through inactive original epithelium as masses of polyhedral cells. ×140.

FIG. 14.—Prostatic duct from 10-month-old guinea pig, castrated and treated with estrogen for 3½ months. Upper surface of duct is lined by alkaline phosphatase-active metaplastic epithelium with precornified squamous superficial layers; lower surface of duct is lined by alkaline phosphatase-inactive original epithelium. ×175.

FIG. 15.—Seminal vesicle of 8-month-old rabbit, castrated and treated with estrogen for 4½ months. Note alkaline phosphatase-positive metaplastic epithelium and basal cells, negative original surface and glandular epithelium. ×290.

FIG. 16.—Prostatic alveolus of 12-month-old intact rabbit, treated with estrogen for 4½ months. Note alkaline phosphatase-active metaplastic growths occluding lumina, in contrast to inactive original epithelium. ×270.

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Effect of Colloidal Au^{198} on the Growth Cycle of Leukemic Cells and on the Survival of Their Host*

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It is the consensus (3) that lymphatic leukemia in human beings and the analogous condition in mice have the same growth pattern. The following successive stages may be distinguished: (*a*) formation of free malignant cells, presumably in the lymphatic system, by mutation of immature lymphocytes; (*b*) an asymptomatic stage of leukemic cell multiplication at the site of origin (or inoculation); (*c*) penetration of the leukemic cells into blood and visceral organs, mainly liver, spleen, and lymph nodes, characterized by illness, and terminated by death. In experimental mouse leukemia the interval between inoculation and death is used as an indicator for a quantitative assay of chemotherapeutic or radiotherapeutic agents. We have attempted to supplement the data of this method about the effect of treatment on the host (survival) by studying its effect on leukemic cells in various stages of their growth cycle.

MATERIALS AND METHODS

Animals.—Mice of Akm strain were supplied by Carworth Farms or bred in our animal room. Animals of 20 to 25 gm. were used.

Leukemic material.—Strain Ak4[1] was carried out in successive intraperitoneal transfers of leukemic cells, and in inoculated mice the cells were counted (*a*) in the peritoneal fluid which was withdrawn with capillary pipettes, (*b*) in the blood which was obtained by cutting the tail (without any compression of tissue), and (*c*) in the spleen (ground and suspended in a convenient amount of 0.85 per cent NaCl solution).

Method.—Mice of each series were inoculated with the requisite number of leukemic cells from the spleen. At various intervals after inoculation, peritoneal fluid and blood were withdrawn; counts of total number of cells and of percentage of leukemic cells were made in each specimen.

Cell counts.—The peritoneal fluid, blood, or suspension of spleen cells was diluted with 3 per cent acetic acid, and their total number per cubic millimeter was determined in a counting chamber. For differential counts, smears of undiluted material were stained with Wright's stain. The technic of standardization of the cell suspension for inoculation has been fully described elsewhere (4).

Mitotic frequency.—Smears from liver, spleen, and lymph nodes were stained with Aceto-orcein, and the frequency of mitoses was recorded.

RESULTS

Growth cycle of leukemic cells in untreated mice.—Table 1 illustrates the fate of intraperitoneally inoculated leukemic cells surveyed daily by parallel study of the peritoneal fluid and of the blood in 30 animals. In specimens withdrawn after 24 hours it was noted that leukemic cells had multiplied in the peritoneal fluid during this period; the fluid contained 10,000 to 40,000 leukemic cells per cubic millimeter, and, since the average amount of fluid in the cavity was about 0.2 cc., the total number of leukemic cells was about 2–8 millions (2–8 times higher than their number in the inoculum); very few leukemic cells (and, in some mice, none) were found in the blood. The total amount of the peritoneal fluid, the total cell count, and the percentage of leukemic cells in the fluid rose rapidly during the first 4 days, while the cell counts in blood remained very low and did not change significantly during the same period. Between the fifth and the sixth days there was little change in the amount of peritoneal fluid, but the majority (about 65 per cent) of abundantly proliferated leukemic cells disappeared suddenly from the fluid, while, simultaneously, large numbers of these cells appeared in the blood. During the next 2 days (sixth and seventh after inoculation), their number increased in the blood at a much higher rate than that previously observed in the fluid, and this

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[1] We appreciate the courtesy of Dr. H. E. Skipper, Southern Research Institute, Birmingham, Ala., who supplied us with the strain Ak4, and of Dr. J. H. Burchenal who provided breeders for our colony of Akm mice.

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trend was arrested only by the death of the animals occurring on the sixth, seventh, and eighth (rarely the ninth) days after inoculation. During these last days, the amount and the cellular content of the fluid remained almost stationary. At autopsy, liver, spleen, and lymph nodes were found infiltrated with numerous leukemic cells. These cells showed a much higher percentage of mitoses than leukemic cells in the peripheral blood.

of leukemic cells from the standard source (spleen) presented in Table 1. This comparison outlined the following growth features: (*a*) After inoculation of leukemic cells from the fluid, the significant rise in the blood occurred later (after 7 days) and remained on a considerably lower level (highest average, 51,000 cells) than after inoculation of the same dose of spleen cells. The mildness of the condition induced in series A, Table 2, is illustrated by longer survival of mice, as compared to those

TABLE 1

GROWTH CYCLE OF 1,000,000 LEUKEMIC CELLS INOCULATED INTRAPERITONEALLY INTO AKM MICE

DAYS AFTER INOCULATION	NO. OF MICE	PERITONEAL FLUID Total count (cells/c mm×10³)	Per cent leukemic	BLOOD Total count (cells/c mm×10³)	Per cent leukemic
1	30	44(26–68)	32(23–41)	10(7–13)	3(0–4)
2	30	74(34–116)	62(52–74)	22(18–32)	19(6–15)
3	30	90(74–122)	68(55–79)	19(13–29)	10(3–17)
4	30	124(61–170)	80(54–92)	23(10–32)	14(2–19)
5	30	66(59–82)	51(29–62)	70(56–103)	56(25–78)
6	21	71(58–88)	48(33–58)	164(100–242)	66(59–80)
7	12	60(55–66)	39(21–64)	175(120–210)	80(78–83)
8	2	58(56–61)	46(24–69)	173(165–181)	79(73–80)

Note: For each group average and range of results are indicated.

TABLE 2

GROWTH CYCLE IN THE BLOOD OF MICE OF LEUKEMIC CELLS TAKEN FROM VARIOUS SOURCES (PERITONEAL FLUID OR SPLEEN) AND INJECTED INTRAPERITONEALLY IN VARIOUS DOSES

	SERIES A 1,000,000 CELLS FROM PERITONEAL FLUID			SERIES B 10,000 CELLS FROM SPLEEN			SERIES C 10,000,000 CELLS FROM SPLEEN		
DAYS AFTER INOCULATION	No. of mice	Total count (cells/c mm ×10³)	Per cent leukemic	No. of mice	Total count (cells/c mm ×10³)	Per cent leukemic	No. of mice	Total count (cells/c mm ×10³)	Per cent leukemic
2	20	8(6–11)	2(0–4)	20	7(3–10)	9(6–15)	20	16(14–22)	18(8–36)
4	20	12(8–17)	7(4–10)	20	18(12–29)	16(10–31)	20	70(57–85)	67(54–96)
6	20	20(13–26)	16(4–28)	20	56(26–72)	69(63–84)	4	98(93–110)	86(65–100)
8	9	50(15–98)	17(11–26)	15	73(31–101)	58(34–79)	3	120(96–168)	88(74–91)
11	4	51(28–94)	19(6–38)	4	68(49–109)	67(54–81)		mice all dead	

Note: For each series average and range of results are indicated.

In order to analyze the factors directing the relationship between the growth of leukemic cells and the condition of their host, we have studied comparatively the rate and the level of multiplication of leukemic cells in the blood after inoculation of very large (10,000,000) and very small (10,000) numbers of leukemic cells from the spleen or of a standard number (1,000,000) of cells from another source (peritoneal fluid). In some animals taken at random, the cellular content was recorded both in the blood and in the peritoneal fluid; in all cases a drop in the cell number in the peritoneal fluid coincided with a rapid increase in the number of cells in the blood. For this reason, Table 2 contains only the data on blood obtained in these experiments. They are compared with the data on the growth cycle of a standard number (1,000,000)

of Table 1. (*b*) Similar changes in the results were obtained by varying a quantitative factor, i.e., by substituting the inoculum of 10,000 spleen cells (series B, Table 2) for that of 1,000,000 spleen cells (Table 1). Vice versa, the inverse change of this quantitative factor, i.e., the increase of the inoculum up to 10,000,000 cells has shortened the "aleukemic" stage of leukemic cell growth and the life span of inoculated mice. Moreover, it was found in additional experiments that even the increase of the inoculum up to 50,000,000 cells was followed by an aleukemic stage; this stage, however, lasted only 3 days and was followed by a very steep rate of increase of cell number in the blood and by rapid death. (*c*) The blood of the majority of mice of series A and B, Table 2, contained, at death, considerably lower numbers of leukemic

cells than that of the mice described in Table 1 or of series C, Table 2. Nevertheless at autopsy the organs (liver and spleen) of all mice dying of leukemia showed a similar picture. These findings indicate that not the invasion of blood with leukemic cells, but leukemic invasion of organs was the cause of death from leukemia. (*d*) The maximum blood cell count in mice inoculated with 10,000 cells was 109,000 cells, and in those inoculated with 10,000,000 cells it was 168,000 cells. Thus, this concentration was not exactly proportional to the inoculum, but was limited apparently by some regulating mechanism.

to the interval between inoculation and treatment, and (*c*) it was directly proportional to the dose of Au^{198}. Thus, the effect depended on the ratio "amount of radioactivity per leukemic cell in a unit of time," and it was due to the direct effect of radioactivity on the agent of the leukemic condition, i.e., the leukemic cell.

This effect was further analyzed by studying comparatively the growth cycle of leukemic cells in treated mice and in controls (Charts 1 and 2). Table 4 shows the importance of the time factor (early treatment) for reducing the concentration of leukemic cells in the blood. While series B

TABLE 3

EFFECT OF VARIOUS DOSES OF RADIOACTIVE COLLOIDAL GOLD ON SURVIVAL OF MICE INOCULATED WITH VARIOUS NUMBERS OF LEUKEMIC CELLS AND TREATED AT VARIOUS INTERVALS AFTER INOCULATION

No. of series		No. of inoculated leukemic cells	Interval between inoculation and treatment (days)	Dose of intravenously injected gold (mc.)	Survivors/nonsurvivors Days after inoculation 8	10	12	Notes
1	Control	100,000			8/2	3/7	2/8	
	A	100,000	2	0.45	9/1	9/1	8/2	
2	Control	100,000			6/4	4/6	1/9	
	A	100,000	2	0.45	10/0	10/0	6/4	
	B	100,000	2	2×0.45	10/0	10/0	3/7	The interval between 2 injections was of 3 days
3	Control	100,000			7/3	5/5	2/8	
	A	100,000	3	0.3	10/0	8/2	7/3	
	B	100,000	3	0.3	10/0	8/2	6/4	Simultaneous intraperitoneal injection of 0.1 ml. citrated blood from Akm mice
4	Control	100,000			5/5	2/8	0/10	
	A	100,000	5	0.45	20/0	15/5	5/15	
5	Control	1,000,000			8/2	4/6	1/9	
	A	1,000,000	3	0.5	20/0	20/0	12/8	
6	Control	1,000,000			6/4	4/6	2/8	
	A	1,000,000	3	0.5	10/0	10/0	3/7	
7	Control	1,000,000			4/6	0/10	0/10	
	A	1,000,000	5	0.4	8/2	5/5	3/7	
	B	1,000,000		0.4	7/3	3/7	3/7	0.5 ml. citrated blood as above
8	Control	1,000,000			4/6	1/9		
	A	1,000,000	5	0.5	9/1	5/5	5/5	
	B	1,000,000		0.5	6/4	4/6	3/7	0.5 ml. citrated blood as above

Effect of radioactive colloidal gold on leukemic animals and leukemic cells.[2]—The object of our radiotherapeutic experiments was to assay the effect of Au^{198} on leukemic mice under conditions imitating as closely as possible those of clinical treatment, which aims to reach leukemic cells in the blood and in visceral organs. For this reason, the intravenous route was used uniformly for administration of Au^{198}. In the first series of experiments illustrated by Table 3, we have investigated the effect of treatment with radiogold on the survival of mice. It is evident from Table 3 that (*a*) this treatment with doses of 0.3–0.5 mc. prolonged invariably the life span of leukemic mice, (*b*) this effect was inversely proportional to the number of inoculated cells and

which received late treatment apparently escaped the effect of radiation and showed a high number and proportion of leukemic cells in the blood, the majority of animals treated early (series A and the mice of Charts 1 and 2) survived 10 days or longer and developed true leukopenia. Charts 1 and 2 indicate by comparison with Table 1 that the reduction in the number of leukemic cells was considerably less marked in the fluid (high proportion of leukemic cells) than in the blood. In several surviving mice the visceral organs presented a normal picture upon gross and microscopic examination. The proportion of large monocytes in the blood of these animals was higher than in untreated mice (10–42 per cent instead of 1–10 per cent). Their peritoneal fluid contained numerous macrophages often studded with the debris of leu-

[2] Radioactive colloidal gold was supplied by Abbott Research Laboratories, Dr. D. L. Tabern, North Chicago, Ill.

kemic cells. The proportion of polymorphonuclear leukocytes and lymphocytes was very low in untreated mice (0–23) but it increased (32–64) after a regression in treated surviving mice. Thus, the treatment with colloidal Au^{198} (*a*) produced a striking effect on leukemic cells in the blood and the organs (less in the fluid), (*b*) elicited a reaction of reticulo-endothelial elements, and (*c*) contributed to maintenance of normal leukopoiesis in hematopoietic organs.

Effect of Aminopterin[3] *on leukemic mice and leukemic cells.*—High toxic doses (single dose of 5 mg/kg or two doses of 2.5 mg/kg) or repeated small doses (0.004 mg/kg, six daily injections) were tested intraperitoneally for their effect on leukemic cells. The effect of therapeutic doses on the survival of leukemic mice was studied extensively by Burchenal *et al.* (1) and Skipper *et al.* (10). Our results, recorded in Table 5, reveal that as early as 12 hours after the intraperitoneal injection of aminopterin the total number of cells in the peritoneal fluid, as well as the percentage of leukemic cells, was sharply reduced. This reduction was progressive and affected leukemic cells and leukocytes at about the same rate until the death of the animal, with leukopenia in the blood and fluid (there were only traces of peritoneal fluid in the mice after the seventh day). Two (10 per cent) mice treated with two doses of 2.5 mg/kg did not exhibit leukopenia and died of leukemia. However, treatment with 0.004 mg/kg doses affected leukemic cells more than leukocytes and induced almost complete disappearance of leukemic cells in all mice, as well as an increase in the number of polymorphonuclear leukocytes and lymphocytes after initial depression. It may be

[3] We appreciate the courtesy of Lederle Laboratories, Pearl River, N.Y., in supplying us with aminopterin.

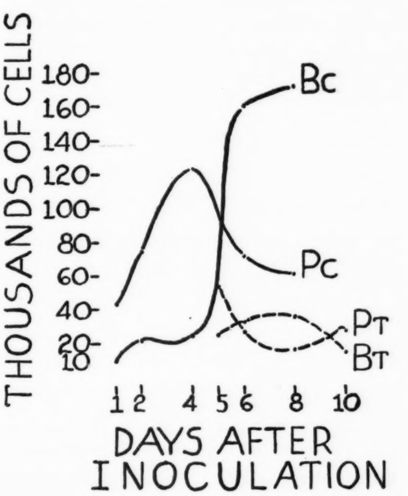

CHART 1.—Total cell counts in the peritoneal fluid and in the blood of leukemic mice, untreated (controls), or treated with radiogold.

Pc = Cell counts in the peritoneal fluid of controls.

Bc = Cell counts in the blood of controls.

PT = Cell counts in the peritoneal fluid of treated mice.

BT = Cell counts in the Blood of treated mice.

Each graph represents an average of 30 mice.

In all mice the treatment consisted of a single dose of 0.4 mc. Au^{198} injected intravenously 3 days after inoculation.

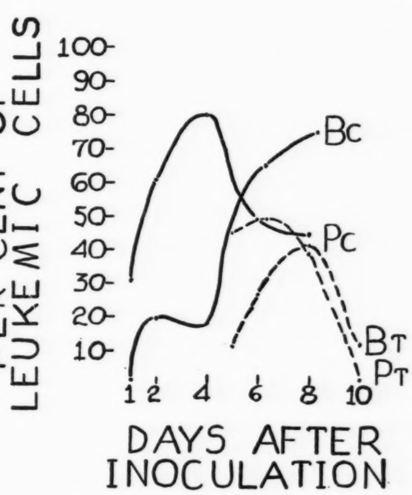

CHART 2.—Percentage of leukemic cells in the peritoneal fluid and in the blood of mice, untreated (controls), or treated with radiogold.

Pc = Per cent leukemic cells in the peritoneal fluid of controls.

Bc = Per cent leukemic cells in the blood of controls.

PT = Per cent leukemic cells in the peritoneal fluid of treated mice.

BT = Per cent leukemic cells in the blood of treated mice.

TABLE 4

EFFECT OF RADIOACTIVE COLLOIDAL GOLD (INJECTED INTRAVENOUSLY AT DIFFERENT INTERVALS AFTER INOCULATION OF 1,000,000 SPLEEN LEUKEMIC CELLS) ON BLOOD OF AKM MICE

	Series A*			Series B*		
		Blood			Blood	
Days after inoculation	No. of mice	Total count (cells/c mm×10³)	Per cent leukemic	No. of mice	Total count (cells/c mm×10³)	Per cent leukemic
5	20	14(10–16)	15(0–29)	10	14(4–31)	41(14–74)
6	20	29(15–46)	46(20–58)	10	22(12–32)	45(26–93)
8	16	32(24–58)	64(11–85)	7	86(17–108)	49(18–67)
10	7	17(4–22)	9(3–15)	5	80(11–159)	91(86–100)

Note: Average and range of results are indicated for each group.

* The interval between inoculation and treatment was: series A, 2 days and series B, 4 days.

concluded that high doses of aminopterin affect leukemic cells and leukocytes in the peritoneal fluid, in the blood, and in the hematopoietic organs, while the latter may be spared by the use of repeated small doses.

Effect of combined treatment with Au^{198} and aminopterin.—Series A and B of Table 6 showed that injection of Au^{198} prolonged the survival of leukemic mice treated with toxic doses of aminopterin (as compared to series A and B of Table 5 showing the effect of the same doses of aminopterin alone). The combination of one small dose of aminopterin for three successive days and a subsequent injection of colloidal Au^{198} was as successful as six injections of the same dose of aminopterin. The additive cytotoxic effect of the two agents on leukemic cells is demonstrated by comparison of Tables 4–6.

TABLE 5

EFFECT OF AMINOPTERIN INJECTED INTRAPERITONEALLY IN VARIOUS DOSES, AT VARIOUS INTERVALS AFTER INTRAPERITONEAL INOCULATION OF 1,000,000 LEUKEMIC CELLS

	Series A, Single dose of 5.0 mg/kg 3 days after inoculation					Series B, Two doses of 2.5 mg/kg on the 2d and 5th days			Series C, Six doses of 0.04 mg/kg daily beginning the 2d day		
		Peritoneal fluid		Blood			Blood			Blood	
Days after noculation	No. of mice	Total count (cells/c mm $\times 10^3$)	Per cent leukemic	Total count (cells/c mm $\times 10^3$)	Per cent leukemic	No. of mice	Total count (cells/c mm $\times 10^3$)	Per cent leukemic	No. of mice	Total count (cells/c mm $\times 10^3$)	Per cent leukemic
3*	20	12(9–16)	24(10–37)	13(7–24)	3(0–8)	20	10(4–15)	14(9–24)	20	17(12–20)	13(7–21)
5	16	8(3–17)	20(2–42)	10(4–14)	16(2–24)	20	14(5–23)	6(2–11)	20	8(4–11)	3(0–8)
7	2	16(12–22)	24(11–40)	5(4–5)	13(4–24)	12	16(11–22)	8(5–21)	16	8(4–12)	5(3–7)
9	all mice dead					2	16(12–28)	69(58–80)	6	9(4–15)	3(2–8)

Note: Average and range of results are indicated for each group.
* Samples taken 12 hours after treatments.

TABLE 6

EFFECT OF COMBINED TREATMENT WITH AMINOPTERIN AND RADIOACTIVE COLLOIDAL GOLD ON LEUKEMIC CELLS IN THE BLOOD (AFTER INOCULATION OF 1,000,000 CELLS)

	Series A, Aminopterin 2.5 mg/kg the 3d day*			Series B, Aminopterin 2.5 mg/kg the 2d and 4th day*			Series C, Aminopterin 0.04 mg/kg the 2d, 3d, and 4th days*		
Days after inoculation†	No. of mice	Total count (cells/c mm $\times 10^3$)	Per cent leukemic	No. of mice	Total count (cells/c mm $\times 10^3$)	Per cent leukemic	No. of mice	Total count (cells/c mm $\times 10^3$)	Per cent leukemic
5	10	8(3–15)	14(9–26)	10	8(5–11)	28(6–58)	20	7(5–12)	7(2–16)
7	6	3(2–5)	6(0–10)	8	9(3–14)	7(2–14)	16	8(4–13)	6(3–11)
9	2	4(3–7)	3(4–11)	3	2(1–2)	9(2–19)	10	15(13–18)	4(0–11)

Note: Average and range of results are indicated for each group.
* 0.4 Radioactive colloidal gold given on the fifth day.
† Samples taken 12 hours after treatments.

DISCUSSION

It has been emphasized (2) that the peritoneal cavity is an integral part of the lymphatic system. Thus, the inoculation of leukemic cells into the peritoneal cavity is biologically similar to the first growth stage of leukemic cells originating in the lymphatic system. Therefore, we may presume that the mechanism of cell distribution between the peritoneal cavity and blood during the aleukemic stage of mouse leukemia is similar to that regulating the aleukemic stage of human leukemia. Our studies on mice demonstrated that inoculated leukemic cells proliferated slowly during the first 24 hours in the peritoneal cavity and at an increasingly higher rate during subsequent days, reaching their maximum concentration in the peritoneal fluid on the fourth day. During this period they penetrated only in very small numbers into the blood. However, on the fifth day the situation was suddenly reversed, and the peritoneal fluid appeared depleted of the majority of leukemic cells, while at the same time they overflowed into blood, producing the condition of "true leukemia." This phenomenon can be interpreted as a sudden change in the condition of either the leukemic animal or the leukemic cells or of both. We may presume that during the first growth stage a normally existing regulating mechanism allowed only a slight "leakage" of leukemic cells from the peritoneal space into blood and that this mechanism failed to function when the cells attained a high level in the peritoneal fluid. It is also possible that leukemic cells growing in the fluid reached at the critical day a new stage in their biological development (surface and shape changes, higher mobility), allowing their escape

into blood. The first interpretation is supported by the finding (Table 1) that, after the sudden escape of numerous leukemic cells from the cavity, the total number of cells in the fluid and the percentage of leukemic cells remained astonishingly constant in all mice, in spite of the presence of numerous mitoses. This observation suggested that the change in the regulating mechanism allowed the cells to overflow from the fluid into the blood as soon as their concentration in the fluid reached a certain level, much lower than the "threshold" observed during the first days after inoculation. It is reasonable to assume that the condition of lymphatic vessels was affected by qualitative and quantitative changes in lymphocytes and that an overwhelming proportion of abnormal elements in the blood impaired, in the later stage, the nutrition of the vessels and changed the composition of blood plasma.

For the second interpretation, there is direct evidence that leukemic cells in the blood and organs possessed a higher growth potency than in the peritoneal fluid. The cell multiplication in the blood proceeded (after 5 days) at a much higher rate and reached a much higher concentration per cubic millimeter. Since the total volume of blood was much higher than the volume of fluid and, moreover, numerous leukemic cells penetrated from the blood into the organs, it is evident that by passing from the fluid into the blood leukemic cells acquired a strikingly high growth potency. This is confirmed by the observation that 1,000,000 cells from peritoneal fluid (series A, Table 2) induced milder leukemia than the same number of cells from the spleen (Table 1). We call attention to a similar observation that tumor cells from subcutaneous implants acquire a higher growth potency when they are cultured as free cells in the peritoneal fluid (4). The above data suggest that in the growth cycle the leukemic cell passes through three stages in growth potency, corresponding to the penetration of cells from the fluid into blood and from the blood into liver and spleen.

It may be concluded that changes in the distribution of leukemic cells between peritoneal fluid, blood, and organs reflected both changes in the regulating mechanism of the host and in the biological characteristics of the leukemic cells.

Series B and C, Table 2, showed that a tenfold increase of the inoculum reduced the aleukemic period but did not eliminate it; similarly, a tenfold decrease only delayed but did not eliminate the stage of true leukemia. Moreover, the maximum cell concentration per cubic millimeter in the blood was not much higher in the former experiments (168,000) than in the latter experiment (109,000). These findings suggest the existence of a "threshold" for leukemic cells in the blood, regulating the passage of leukemic cells from the blood into the organs. It was the abundant invasion of liver and spleen with leukemic cells and not their high blood level that was found in all mice which died of leukemia. This suggests that this final stage of leukemic cell growth brought about death.

Comparison of the leukemic growth cycle in untreated and treated mice showed the importance of cell counts in the fluid and the blood, as well as the autopsy findings, in the interpretation of therapeutic results. The first trial of radioactive colloidal gold in mouse leukemia was carried out by Hahn *et al.* (8). A small but significant extension of survival of mice inoculated with 10,000 leukemic cells was obtained by intraperitoneal injections of 0.1–0.35 mc. of Au^{198}. Sheppard, Wells, Hahn, and Goodell (9) found that intravenously injected colloidal gold is rapidly deposited in the liver and the spleen, and it seemed more reasonable to attempt the treatment of leukemic invasion of these organs by intravenous injection of this agent. The data on survival of mice treated by this route at various intervals after inoculation of 1,000,000 cells indicated (Table 3) that Au^{198} showed specific characteristics of an agent increasing survival time in leukemia by direct action on the leukemic cells. The therapeutic effect of the agent was distinctly correlated with the size of the inoculum and the extent of cell multiplication before treatment. However, cell counts (Table 4) revealed some other effects of Au^{198} on the leukemic condition: (*a*) The critical rise of leukemic cell concentration occurred in mice treated late (series C, Table 4) but not in those treated early. This phenomenon may be ascribed to the improvement of the regulating mechanism after treatment. Moreover, cell counts in the peritoneal fluid (Chart 1) showed that an initial slight reduction of cell number was followed after 5 days by the expected drop. However, the phenomenon was not paralleled in any mouse by a corresponding increase of cell number in the blood. It seems that cells leaving the peritoneal cavity did not get into the blood and remained in the lymph nodes. Thus, the invasion of the blood was at least partially prevented by treatment. (*b*) The average number of leukemic cells in the blood of mice treated with Au^{198} did not vary significantly between the fourth and the seventh days, indicating that multiplication of cells in the blood was limited (scarcity of mitoses). Some cases of late treatment (series B, Table 4) were terminated by leukemia and death. Moreover, in two cases of this series and in most mice of other series the initial cyto-

penia was followed by the reappearance of large numbers of polymorphonuclear leukocytes and lymphocytes in the blood and, more significantly, by an increase in number of large monocytes in the blood and the fluid. Thus, leukopoiesis was not affected by radiation, and, moreover, there was a stimulation of reticulo-endothelial elements previously recorded in mice treated with this agent (5). In both series shown in Table 4 there were large individual variations in the response to treatment. The range of variations was particularly large in mice treated after 4 days (series B). Since the disappearance of leukemic cells was constantly associated with an increase of the proportion of large monocytes, it may be presumed that variations in therapeutic results were due to individual differences in stimulation of the reticulo-endothelial system by radiogold.

The effect of aminopterin on leukemic cells and leukocytes in the blood and peritoneal fluid can be fully described as cytotoxic: rapid decrease in cell number with or without subsequent regeneration of blood cells. Peritoneal fluid and blood were equally affected, owing to rapid diffusion of the injected compound. High doses reduced the numbers of leukocytes and of leukemic cells at a similar rate, but repeated small doses affected the number of leukemic cells more than that of leukocytes, which was presumably caused by the rebuilding of leukocytes (mainly polymorphonuclears) by hematopoietic organs. Thus, this method of treatment has the value of a chemotherapeutic method, since it allowed aminopterin to exert its cytotoxic action on all cells in fluid and blood, but spared the normal cells of bone marrow. There is some indication that cell distribution regulating mechanisms were not improved by this treatment, since (*a*) simultaneous cell decrease in the fluid and increase in the blood occurred in mice treated even with high doses and (*b*) leukemic cells were abundant in the organs even if they were not numerous in the blood. Individual variations in treated animals were not marked, and thus there was no indication that the defense mechanisms of the host were stimulated by the treatment. The powerful cytotoxic effect of aminopterin has been demonstrated *in vitro* by Gunz (7). Lack of regeneration of blood elements after treatment with high doses can be interpreted in terms of the anti-anabolic effect of aminopterin described by Gubner (6).

The combined treatment with aminopterin and colloidal Au^{198} revealed a synergism in their action on leukemic cells, i.e., this treatment reduced the number of cells in the fluid and in the blood to a much greater degree than treatment with either of these compounds alone. This agrees with our conception of the cytotoxic nature of both agents. Unexpectedly, the administration of Au^{198} prolonged the survival of mice treated with toxic doses of aminopterin. It may be presumed that the stimulating action of colloidal gold on reticulo-endothelial elements, resulting in better "scavenging" from the blood of dead cells and cell debris, has compensated for the anti-anabolic inhibition by aminopterin.

The data reported here represent only a first attempt to apply the analysis of the growth cycle of leukemic cells to the assay of anti-leukemic agents. These data suggest that therapeutic research in leukemia should aim toward restoration of the normal blood picture not only by destruction of leukemic cells, but also by restoration of the normal regulatory mechanisms of the host.

SUMMARY AND CONCLUSIONS

1. Various numbers of leukemic spleen cells were inoculated intraperitoneally into Akm mice. Blood and, in several series, peritoneal fluid were withdrawn from these mice repeatedly, at various intervals. The total number of cells and the percentage of leukemic cells were counted in each specimen, and the results were reviewed in tables illustrating the growth cycle of leukemic cells.

2. It was found that after the intraperitoneal inoculation of 1,000,000 of these cells they multiplied in the peritoneal fluid and reached their maximum number on the fourth day, while only few leukemic cells appeared in the blood during this period (aleukemic stage). However, on the fifth day, the number of leukemic cells in the peritoneal fluid suddenly decreased, whereas leukemic cells appeared at that time in blood in large numbers (true leukemia) and continued to multiply at a very high rate, invading the spleen, liver, and lymph nodes, until the death of the animal.

3. The reduction in size of the inoculum (to 10,000 cells) or the use of 1 million cells from peritoneal fluid delayed the change of the aleukemic stage into leukemia and the death of the animal; increase of the inoculum to 10,000,000 or more shortened the aleukemic stage and reduced the life span of the animal. Thus, the three stages of growth occurred in all inoculated animals. The occurrence of death was not correlated with the level of leukemic cells in the blood but with the invasion of organs by these cells.

4. Evidence is presented to show that during the three stages of the growth cycle the growth potency of leukemic cells increased. However, the inversion of cell distribution initiating the second and the third stages was induced apparently by

disturbances of the regulatory mechanism of the host.

5. Intravenous injection of colloidal Au^{198} considerably prolonged the survival of leukemic mice and reduced the number of leukemic cells in the blood and the organs (slightly in the fluid). It is concluded that the therapeutic effect of this agent is due (*a*) to its direct cytotoxic action on the leukemic cell, (*b*) to improvement of the regulating mechanism of the host, and (*c*) to stimulation of reticulo-endothelial elements.

6. Treatment with repeated small doses of aminopterin drastically reduced the cellular content of the blood and the peritoneal fluid but allowed the replacement of destroyed leukocytes by production of the hematopoietic organs and, therefore, extended the survival of mice.

7. Combined treatment with Au^{198} and aminopterin demonstrated a synergistic cytotoxic effect on leukemic cells. Moreover, mice survived longer than mice treated with aminopterin alone. This is attributable to the compensation of the anti-anabolic effect of aminopterin by stimulation of reticulo-endothelial elements with colloidal gold.

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The Effect of Heat Inactivation on Precipitation of Serum Proteins by Means of Sodium Chromate in Sera of Normal and Cancerous Subjects

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The question whether the proteins in the blood serum of cancer patients differ from those of normal individuals has been the subject of numerous investigations (2, 5, 7). Various analytical procedures have been devised to demonstrate such a difference in one or another of its aspects. The results obtained in the analysis of native cancer serum are usually compared to those of native normal serum; however, an occasional method has been proposed in which determinations are made on serum in its native and in its heat-inactivated state, and the relative change in the same serum is considered. This has the advantage of eliminating to some extent individual characteristics, the absolute values of the serum being of minor importance.

The following is a summary of results obtained by the analysis of normal and cancer sera by such a method, previously described (1, 4, 6). The procedure has no claim to greater practical simplicity or to sharper differentiation between normal and cancer sera than other proposed methods. The results are reported here to describe some conclusions reached in its application which may be of use in the evaluation of other, similar procedures.

METHODS

Increasing amounts of sodium chromate in dilute propionic acid solution were added to blood serum, and the height of the precipitated protein sediment was measured in each case. This was done with native serum and simultaneously with the serum after heat inactivation. The relative position of the sedimentation zones was considered. A heat-inactivated serum is in general more stable with respect to sodium chromate than the same serum in its native state, and it thus requires a higher chromate concentration to produce a comparable degree of sedimentation. This stability difference between the native and the inactivated state is less in cancer sera and may even be completely absent.

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Blood was obtained, preferably several hours after the last meal, and serum was taken off the clot after approximately 6 hours at room temperature. No preservatives were added. Two stock solutions were used: (*A*) 0.1 M sodium chromate (2.34 gm. $Na_2CrO_4 \cdot 4\,H_2O$ with water to 100 ml.); and (*B*) 0.1 M propionic acid.

A series of solutions with increasing sodium chromate concentrations was made as follows: 5.0 ml. of solution *A* were diluted to 100 ml. with solution *B*. The same procedure was followed with 5.5 ml. of solution *A*, 6.0 ml. of A, etc., to 8.0 ml. of *A*.

Two rows of six flat-bottomed Pyrex tubes (7 × 100 mm.) were placed in a suitable stand in which the tubes hung vertically on their rims. In each tube was placed 0.2 ml. of the native serum. The six tubes in the rear row were gathered, covered, and placed for 30 minutes in a constant temperature water-bath at 56° C. They were then cooled and replaced in the stand.

2.0 Ml. of the appropriate sodium chromate-propionic acid solution was added to each tube. The contents were mixed gently by inverting the tubes twice. After the tubes had stood overnight (or for at least 12 hours) at 20° C., the height of the precipitate in each tube was measured (estimated to 0.1 mm.); these results were plotted on graph paper against the corresponding chromate concentration. The difference was then determined between the chromate concentration at which precipitation in the native serum began (by extrapolation to height 0) and the concentration at which sedimentation was equally strong in both sera (intersection of the plots). This expression of the difference in stability of the proteins before and after inactivation of the serum is designated *d* and read in mM sodium chromate per liter (for diagrams see the original article [1]).

The tubes should be kept scrupulously clean in order to prevent the precipitate from adhering to the sides. Between measurements they should be placed in chromic acid cleaning solution, washed, rinsed with distilled water, and dried.

RESULTS AND DISCUSSION

Physical basis of the procedure.—It has been demonstrated (3) that during heat inactivation of human blood serum a decrease of albumin content occurs, probably through adsorption of albumin on a denatured globulin complex, and that this decrease is significantly greater in cancer sera. Although the precipitation of a heat-denatured albumin-globulin mixture by an oppositely charged polyvalent ion is a complicated phenomenon, involving such insufficiently clarified factors as protective action, particle size, and hydration, it seems probable that the difference observed in the precipitation of inactivated normal and cancer serum is due to the presence of larger amounts of an albumin-globulin complex in the inactivated cancer serum which leads to heavier sedimentation at the same chromate concentrations.

Influence of temperature.—The temperature at which the sedimentation takes place is decisive. At low (refrigerator) temperatures the stability difference d is smaller in sera of cancer patients but also in sera of pregnant individuals and generally in sera of normal young women during the intermenstrual period of the cycle. With rising temperatures of sedimentation the stability difference of sera of cancer patients remains relatively smaller, but that of the others approaches the normal value. Typical examples of these conditions are given in Table 1.

These observations suggest that in all these conditions adsorption of a component in the protein complex occurs but that only in cancer serum are the adsorption forces sufficiently strong to prevent release of this component at higher temperatures.

The stability difference in sera of normal young women when measured at low temperature is a function of the phase of the menstrual cycle. The average values of 50 observations in 18 normal individuals are reproduced in Table 2.

Under these conditions (low temperature of sedimentation) the sera of normal young women approach the d value of "normal" serum only at the time of menstruation.

In order to exclude as much as possible divergent influences of conditions other than cancer, measurements of sedimentation were made at 20° C. by placing the tubes in a constant temperature cabinet maintained at that level.

Results in normal and in cancer sera.—Chart 1 represents the frequency distribution of the stability difference d at 20° C. in 230 presumed noncancer cases, in 126 cancer cases, and in 31 cases of pregnancy. Excluded from the noncancer cases were patients who had had recent surgery or irradiation, those known to have fever, and those known to have a positive serum Wassermann reaction, conditions in which the equilibrium of the serum proteins may be disturbed.

Cases of cancer comprised: cancer of the larynx, 4; lung, 2; palate, 1; tongue, 3; pharynx, 2; salivary gland, 1; esophagus, 1; stomach, 6; colon, 7; rectum, 4; pancreas, 3; kidney, 1; bladder, 6; prostate, 2; vulva, 5; vagina, 4; cervix uteri, 38; endometrium, 14; ovary, 11; breast, 7; skin, 2; retroperitoneal sarcoma, 1; leukemia, 1. In the cases reported here analysis was made of specimens obtained before treatment had begun, and classification was deferred until after the diagnosis had been established.

TABLE 1

RELATION BETWEEN STABILITY DIFFERENCE d AND TEMPERATURE OF SEDIMENTATION

Temperature of sedimentation	2° C.	12° C. (d in mM)	22° C.
Normal woman past menopause	0.48	0.64	0.83
Normal woman, 12th day of cycle	0.13	0.54	0.80
Pregnancy, 3d month	0.20	0.34	0.75
Cancer of cervix	0.12	0.18	0.24

TABLE 2

RELATION BETWEEN STABILITY DIFFERENCE d AT 2° C. AND THE PHASE OF THE MENSTRUAL CYCLE

Day of cycle	d in mM
1–5	0.28
6–10	0.11
11–15	0.08
16–20	0.06
21–25	0.21
26–30	0.24
over 30	0.38

Pregnancy cases included: 1st trimester, 7; 2d trimester, 2; 3d trimester, 22 (including 2 in labor).

It is evident that the stability difference between native and inactivated serum with respect to sodium chromate is generally smaller in cancer than in normal sera, although there is some degree of overlapping. An arbitrary reference line is drawn at $d = 0.6$ mM chromate per liter.

Of the four pregnancy cases which show values less than 0.6 mM, three were at term, one in the second stage of labor.

Chart 2 gives the d values for the cases of cancer of organs of the female genital tract and the breast,

separated from other organs. It is notable that 7 out of 38 cases of cancer of the cervix are found within the normal range and that the *d* value decreases in the order: cervix–endometrium–ovary and to generally lower levels in other internal organs. Whether this must be attributed to the difference in the nature of the tissue in which the neoplasm originates, to its connection with the circulatory systems, or, assuming that cancer of the cervix may on the average have been recognized at an earlier stage, to the length of time the tumor has been growing, are at present open questions. It is evident that a number of cases of cancer of the cervix were diagnosed as such, clinically, before altered protein characteristics could be detected in the blood serum by this method.

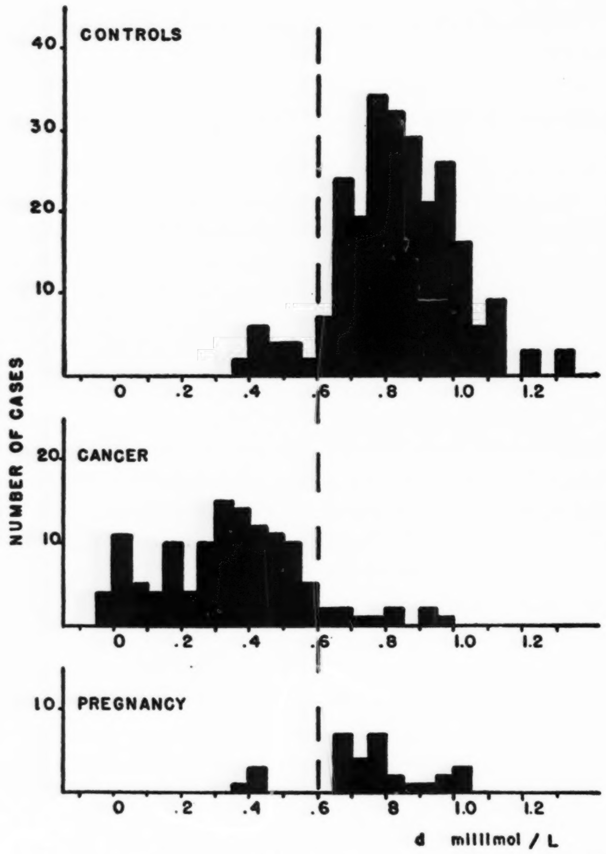

CHART 1.—Frequency distribution of *d* at 20° C. in 230 noncancer cases, in 126 cancer cases, and in 31 cases of pregnancy.

Cancer sera after treatment.—A number of sera were analyzed of patients who had received treatment for cancer and who were considered to be clinically well at the time the analysis was done. Chart 3 gives the results for cancer of the cervix, endometrium, ovary, and breast. In all the cases of cancer of the cervix represented here, therapy had consisted of extensive irradiation; two patients had, in addition, undergone surgery. The cases of cancer of the endometrium had all been treated by surgery. The time that had elapsed since conclusion of the treatment varied from 1 to 12 years.

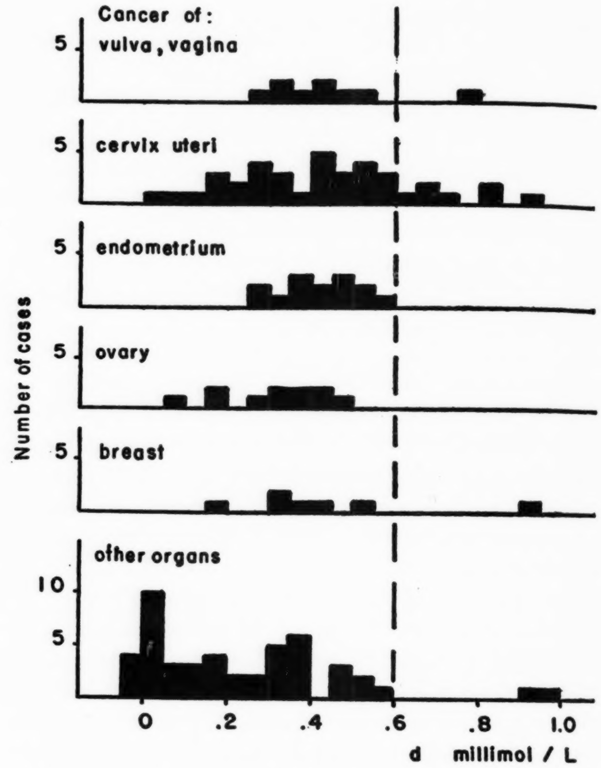

CHART 2.—Frequency distribution of *d* at 20° C. with respect to site of neoplasm.

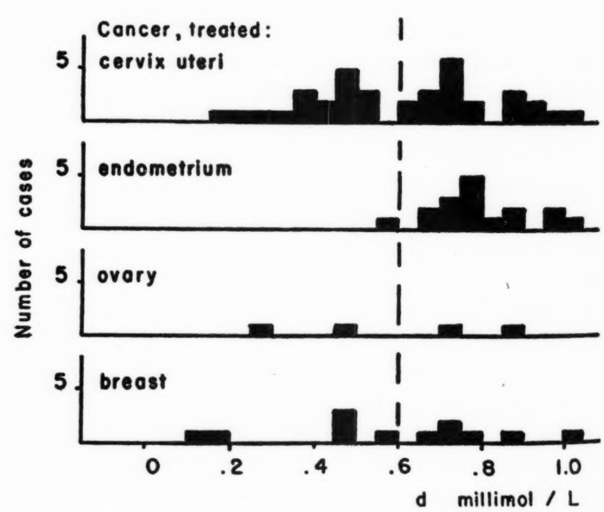

CHART 3.—Frequency distribution of *d* at 20° C. after treatment of cancer patients.

It was found that 17 out of 37 cases of cancer of the cervix still showed the presence of serum proteins different from the normal. In only 1

out of 17 cases of cancer of the endometrium was such the case. No correlation could be established between the chromate value and the time that had elapsed since the conclusion of the treatment, nor between that value and the total amount of radiation administered or the histological evidence of radiation effects in vaginal smears at the time of analysis. The possibility of lymph node invasion in cancer of the cervix was raised as an explanation for the continued abnormality of the serum proteins but remains unproved. In 12 cases of cancer of the breast the results, after treatment, were similar to those of cancer of the cervix. Observations on cancer of the ovary after treatment were too few to warrant a conclusion.

SUMMARY

A method of analysis, in which the difference in stability between the proteins of native and of heat-inactivated blood serum is determined with respect to sodium chromate, was applied to specimens of 230 noncancer cases and of 126 cancer cases. It was confirmed that this difference is significantly smaller in sera of cancer patients. Consideration of the extent of the deviation from normal values in connection with the affected organs showed that the stability difference decreases in the order: cancer of the cervix–endometrium–ovary, and to generally lower values in cancer of other internal organs. Analysis of sera of cancer patients who had received treatment and were considered clinically well showed continued cancer values in 17 out of 37 cases of cancer of the cervix, as against 1 out of 17 cases of cancer of the endometrium.

ACKNOWLEDGMENTS

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Hemolysins in Spontaneous Mouse Breast Tumors as Compared to Those in Normal Mouse Tissue*

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This paper is concerned with the nature of the hemolytic material which occurs in certain tumors, and particularly with the question whether it differs from the hemolytic material found in many normal tissues. The latter, at least in one of its forms, has been shown to have the characteristics of soaps and of lysolecithin-like substances more or less loosely combined with inhibitory material in lysin-inhibitor complexes (7, 9, 10). The soaps and lysolecithin-like substances can be extracted from these complexes with alcohol and are extremely lytic. The complexes themselves can be extracted with saline. Their hemolytic activity is much less than that of the alcohol-extractable substances. Their activity increases if the tissue is incubated at 37° C., probably because loosely combined complexes are formed, by enzymatic action, from complexes in the tissues in which the lysins are more firmly bound to inhibitory material. The activity becomes less or even disappears if the extract is heated to between 60° and 100° C.; this is probably due to denaturation or coagulation effects on protein components of the complexes.

The lytic substances extracted from tumors, on the other hand, vary greatly with the type of tumor used. Two descriptions (3, 4, 14) can be used to illustrate the divergence of opinion. Nonnecrotic tumors (dog sarcoma) contain substances which are the same as the lytic substances of normal tumors, although they occur in greater concentration (14).[1] Spontaneous mammary tumors of the C3H mouse contain lytic material which is not present in normal mouse tissues. The lysin of the tumors is active, producing lysis in an hour or so; it is unstable on standing, is specific for mouse red cells, and is destroyed by heating to 68° C. for 30 minutes (3). Lysins are present in certain human carcinomas (4) but not in normal tissues; these lysins are much less active than those of the mouse tumors, but, like them, are thermolabile (destroyed by heating to 56° C. for 30 minutes).

The essential point is the disagreement as to the occurrence of lytic material in normal mouse or human tissue which some observers find (1, 6, 7, 9, 10, 14) and which others (3, 4) do not find. This may be due to a difference in technic. The tumors of the C3H mouse were extracted rapidly with saline (3), whereas most of the investigators who have found lytic substances in normal tissue have worked with tissue slices or minces, allowed to stand at room temperature or at 37° C. either before the addition of red cells or in actual contact with red cells. It is therefore possible that there are two sets of lytic substances, one peculiar to tumors and unstable, and the other occurring in normal tissues which are allowed to stand or which are pre-incubated. From an operational point of view, indeed, it is convenient first to distinguish between the lysins in fresh saline extracts, the lysins in pre-incubated saline extracts, and the lysins which can be extracted with organic solvents, and then to compare the properties of the hemolytic material, in each category, obtainable from normal tissue and from tumors.

EXPERIMENTAL

OCCURRENCE OF LYTIC MATERIAL IN FRESH SALINE EXTRACTS

Normal mouse lung and spontaneous mouse mammary carcinoma of the C3H mouse were ex-

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[1] Heating extracts containing this lysin to a temperature of 70° C. "usually results in the production of an entirely new set of bodies, which act as anti-hemolysins" (14); it was suggested that the lytic substances may be heat-stable, but the matter was dismissed at that time (1907) as being too involved for further discussion. The situation as regards the heat stability of extracts of both normal tissues and tumors is still unsettled. The lysin obtained from normal tissue slices is destroyed by heating to between 60° C. and 80° C. for 5–10 minutes (1, 6, 7), but complex effects are observed (13). This is probably due to the lysins existing as several lysin-inhibitor complexes, some produced enzymatically from others (9). The enzyme is heat-labile, and the complexes are also heat-labile because of thermal effects on their protein components, but the free lysin which is associated with the complexes, and the lytic component which is extractable with organic solvents, are relatively heat-stable, even when boiled.

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tracted in exactly the same way, i.e., by adding 2 ml. of 1 per cent NaCl per gram of the tissue, mincing with scissors, grinding quite coarsely but without delay in a mortar, and centrifuging at once at 1,500 *g* for 15 minutes. The supernatant fluids were removed and set up with mouse red cells in dilutions by powers of 2. Each system contained 0.2 ml. of the diluted extract and 0.1 ml. of

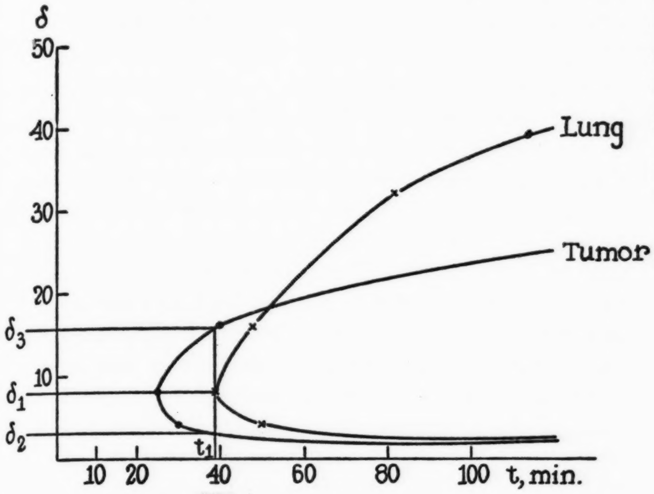

CHART 1.—Typical time-dilution curves for mouse red cells and fresh saline extracts of mouse lung and mouse mammary tumor at 37° C. Ordinate, dilution of extract; abscissa, time in minutes. For further explanation, particularly regarding the zones, see text.

a suspension containing the thrice-washed red cells of 1 ml. of normal mouse blood suspended in 100 ml. of saline. The systems were kept at 37° C. in a water bath, and the times for complete hemolysis were observed.

Concerning the conclusions (*a*) that fresh saline extracts of normal mouse tissue and of mouse tumor both contain lytic substances and (*b*) that the activity in the tumor extracts is usually from 2 to 4 times greater than that in extracts of normal mouse tissue, preliminary experiments with tumor extracts, together with the experience already gained with extracts of normal mouse tissue (9, 10), have shown that the initial concentration of the tissue or tumor extract must be sufficiently great to allow a series of dilutions in powers of 2 to be made, in order to obtain the time-dilution curves shown in Charts 1 and 2. The extracts were accordingly made initially in a concentration of 1 gm. of tissue to 2 ml. of saline. The speed and time of centrifuging are not at all critical, i.e., the extracts can be spun for 10–30 minutes at about 1,500 *g*. Again, as far as the conclusions (*a*) and (*b*) are concerned, substantially the same results were obtained if the dilutions were made before or after centrifuging. The concentration of red cells used in the hemolytic systems was selected because this concentration is optimal for the measurement of the time for complete hemolysis and the obtaining of continuous curves such as those of Charts 1 and 2. The use of suspensions of a much greater concentration (4 or more times greater) results in a failure of the systems to hemolyze (5), presumably because of a shift in the position of the hemolytic zone (see Chart 1), and hemolysis may occur in a single tube or not at all; under such circumstances extracts which contain measurable quantities of lysin appear to be almost, or completely, nonlytic. It is quite likely that this phe-

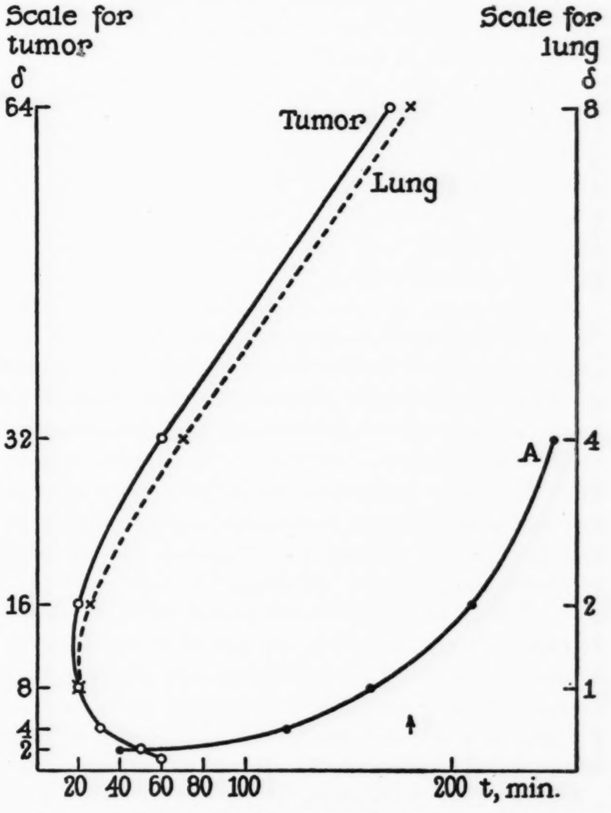

CHART 2.—Time-dilution curves for mouse red cells and supernatant fluids from pre-incubated saline extracts of mouse lung (crosses) and mouse mammary tumor (circles); pH 6.5, 37° C. Scale for dilution of tumor on left ordinate; scale for dilution of lung on right ordinate. Abscissa, time in minutes. Curve *A*, time-dilution curve for supernatant fluid from pre-incubated saline extract of mouse liver at pH 6.5, showing the effect of continued production of lysin. The scale is that for the tumor.

nomenon occurs because absorption of the lysin on the red cell surfaces is a preliminary step in the hemolytic process.[2]

[2] The effect of increasing the concentration of red cells in these systems, as in hemolytic systems in general, is to depress the apparent activity of the lysin. If the concentration of lysin is relatively small (as in the case of extracts of normal tissues), its activity may be reduced to less than that of the asymptotic

Apart from the material used in the preliminary experiments, the results to be described are based on the measurement of the lytic activity of extracts of 53 spontaneous mammary tumors occurring in 46 mice, and of about twice that number of normal mouse tissues. The usual procedure was to measure the lytic activity of the fresh saline extracts at once for the result in Section I and then to use the material remaining for extraction with alcohol, etc., for the results of Section II. The occurrence of the lytic material in pre-incubated saline extracts was investigated in a separate series of experiments, with fifteen tumors and about the same number of normal lungs. Usually, the normal tissues were taken from female mice without tumors or from males, but sometimes they were taken from the tumor-bearers themselves. These variations in technic turned out to be unimportant. The red cell suspension was usually prepared from the blood of normal male mice, but sometimes the blood of females or of tumor-bearing females was used; again the variation in technic is unimportant.

1. The form of the time-dilution curve.—The time-dilution curves for complete hemolysis by fresh extracts of mouse lung, kidney, and tumor are characteristically atypical in that hemolysis occurs at first more rapidly, and then less rapidly, as the concentration of the extract is increased. The time for complete hemolysis thus passes through a maximum corresponding to some particular dilution (Chart 1). Atypical curves such as these, i.e., curves showing "zones" or "prozones," were obtained in 27 out of 33 fresh saline extracts of tumors and in 31 out of 40 fresh saline extracts of normal mouse tissue (32 of lung, 8 of kidney). The remainder—6 tumor extracts and 9 extracts of normal tissue—did not produce lysis in any dilution tested. It should be pointed out, however, that if the two limbs of the curve pass sharply toward the minimum, it is easy to miss the minimum altogether, particularly since the activity of fresh extracts decreases with time (see subsection 3). Similar curves have been found for a number of lysins (8), and observations regarding the hemolytic activity of such lysins are obviously very difficult, or even impossible, to interpret unless the entire curve is plotted. For example, a time t_1, observed for complete hemolysis in a system containing a dilution δ_1 of an extract of lung, may be interpreted as showing either that the extract has more hemolytic activity than a tumor extract (a smaller dilution δ_2 of which also gives complete hemolysis in time t_1), or that the extract has less hemolytic activity than a tumor extract (a larger dilution δ_3 of which also gives complete hemolysis in time t_1). Again, if we test an extract in one arbitrarily selected concentration only, and find no hemolysis, we do not know that we are not in the region of the lower limb of the time-dilution curve and that a *less* concentrated extract would not be *more* active. If we have the two curves in their entirety, however, we can look at their upper asymptotes, from the position of which it appears (in Chart 1) that the lung extract is about 1.5 times as active as the tumor extract, or at the position of the maxima, from which it appears that the tumor extract is a little more hemolytic than the lung extract. The differences, however, are small (the ratio of the hemolytic activities, regardless of how they are measured, is not more than 2 or less than 0.5, and the steps in the dilution series are by powers of 2), and so it can be concluded, in this case, that the hemolytic activity of normal mouse lung and of the mouse tumor are approximately the same. In the case of 25 pairs of curves similar to these in their general form, the extract of tumor and the extract of normal tissue had approximately the same lytic activity in nine cases. In two cases, the extract of normal tissue was more hemolytic than that of the tumor extract tested in the same experiment. In the fourteen other cases, the extract of tumor was more active than the extract of normal tissue (about twice as active in eight cases, about 4 times as active in six cases, and about 8 times as active in two cases).

Since we are dealing with mixtures of lysins and inhibitors, it does not follow that the lung and the tumor contain approximately equal amounts of the same hemolytic material. It is possible, for example, that the tumors contain a smaller concentration of a different and more powerful lysin. A situation which presents so many alternatives could be clarified by showing that there are qualitative differences between the lytic material of normal tissues and that of the tumors; if no such differences can be found, the simplest conclusion will be that normal tissues and the tumors contain the same lysin or mixture of lysins and inhibitors

concentration, and no lysis will occur in the system; if the concentration of the same lysin is relatively great (which may be the situation in the case of the majority of the tumor extracts), the same degree of depression may leave considerable quantities of lysin remaining, and the system will hemolyze. In this way it is possible, by using relatively concentrated suspensions (4 or more times the concentration used here), to be led to the conclusion that there are lysins present in systems containing extracts of tumor, but not in systems containing extracts of normal tissue, although both kinds of system contain the same lysin in different concentrations. This effect of the concentration of the red cell suspension ought to be very pronounced in systems in which there is a preliminary adsorption of the lysin on the cells (see 8, p. 195).

and that variations in hemolytic activity reflect nothing but concentration differences. The remainder of this paper is concerned with the result of unsuccessful attempts to find the qualitative differences referred to.

2. Specificity.—Mouse red cells are more rapidly hemolyzed by fresh extracts of both mouse lung and mouse mammary tumor than are rabbit or human red cells, but, since hemolysis of the latter occurs when the extracts are used in optimum concentration, this should not be interpreted as showing that the lysins are species-specific. The claim that they are has not been substantiated (1, 9) and the differences in the rate of hemolysis in systems containing various types of red cells seem to be merely a reflection of the fact that red cells can be arranged in resistance series (8). In the case of mouse lung and mouse mammary tumor, the series is mouse > man > rabbit > sheep. There are probably many resistance series corresponding to a multiplicity of tissue lysin-inhibitor complexes (1).

3. Instability of the lytic material.—The lytic material of freshly prepared extracts of normal mouse tissue and of mouse tumor is unstable. This can be shown by plotting time-dilution curves or by determining the highest dilution of the material which will produce complete hemolysis in a fixed time, e.g., 60 minutes. The form of the time-dilution curve makes it very difficult to compare results quantitatively, but in most experiments the hemolytic activity of the extracts was reduced to about one-quarter of their initial values when the extracts were allowed to stand for 2 hours at 25° C. No difference has been noticed between extracts of lung and extracts of tumor as regards the rate at which the lytic activity disappears. The situation is complicated by the fact that after an extract has stood for several hours, the effects of standing pass into the effects of pre-incubation, i.e., the disappearance of the unstable lysin initially present is compensated for by the appearance of lytic material which is associated with enzymatic activity in the extract (see below).

4. Agglutination phenomena.—The agglutination of red cells by fresh extracts of mouse tumors (3, 12) occurred frequently in the foregoing experiments. A weaker agglutination occurred with fresh extracts of normal mouse lung and kidney. In the case of both normal tissues and tumors, rabbit red cells were more affected than human red cells, and the latter more than mouse red cells. Generally speaking, agglutination is most marked in the higher concentrations of the fresh extracts; these are the concentrations which produce very slow lysis because of the zone phenomenon, and so the impression is conveyed that agglutination and lysis are reciprocally related, the former occurring when the latter does not, and vice versa.

5. Liquefied tumors.—Many of the larger mouse mammary tumors were found to be black, friable, and apparently necrotic. A brown-black fluid could often be withdrawn; when diluted serially with saline, this fluid was found to be as hemolytic as the most lytic of the tumor extracts. In sections of the tumors it was noted that the black appearance was due to cavities in broken down tumor tissue filled with old hemolyzed blood; it is therefore certain that the lysins of the tumor can produce at least local hemolysis. No special significance can be attached to this, however, for a similar local hemolysis, due to a combination of the effects of stasis and of the lysin of normal (or injured) tissues, is regularly found in hematomas.

Extraction of the brown fluid from these liquefied tumors yields soaplike and lysolecithin-like substances similar to those obtained from the entire tumor. The possibility of their being dialyzable lysins present in addition (14) has not been investigated.

OCCURRENCE OF THE LYTIC MATERIAL IN PRE-INCUBATED SALINE EXTRACTS

Most of the investigators in the field have observed that the supernatant fluids obtained from pre-incubated slices, minces, or homogenates of various normal tissues, as well as of C3H mouse mammary tumor, are hemolytic. The homogenates are prepared as already described (9), 2 ml. of isotonic NaCl being added to each gram of wet tissue; after a few hours of incubation at 37° C., it becomes possible to separate a clear supernatant fluid, the hemolytic activity of which increases with the time during which the pre-incubation is continued. The hemolytic activity of the clear supernatant fluid is found by observing the time required for complete hemolysis by various dilutions of the fluid, in powers of 2, mouse red cells being used in the hemolytic systems. The pH change which occurs in pre-incubated homogenates of mouse liver is much less marked in pre-incubated homogenates of mouse lung and tumor, and it is possible to make comparisons of hemolytic activity at pH 6.5 and after 18 hours of pre-incubation.

When this is done, it is found that the supernatant fluid derived from the tumor homogenate takes shorter times to produce complete hemolysis than does that from the lung homogenate; the following relations between the times for complete hemolysis and the dilution of the supernatant fluid from the lung or tumor homogenates, presented in Table 1, are typical.

The supernatant fluid from the tumor is more lytic than that from the lung, a time for complete hemolysis of 20 minutes being found for a 1 in 8 dilution of the former but for the latter only when undiluted. If the values are plotted on two scales (Chart 2), the one 8 times the other, the two time-dilution curves virtually coincide, except for the values for the dilutions 1 in 1, 1 in 2, and 1 in 4 of the supernatant fluid from the tumor; these fall in a zone (cf. Chart 1). The conclusion, accordingly, is that the difference in the lytic activity found in the two supernatant fluids may be merely a concentration difference. The results of all our experiments with the supernatant fluids of pre-incubated homogenates point to this general conclusion. It can easily be seen, however, that the results of single observations, as contrasted to those obtained when entire time-dilution curves are plotted, can be very misleading, particularly because of the zone phenomena. It should be further noticed that the lysis in these systems is slow and that the form of the time-dilution curves may be distorted because of another phenomenon. This is the continued production of hemolytic material in the supernatant fluid itself when, as in the hemolytic systems under observation, it is kept at 37° C. for many hours. This continued production results in a turning-up of the time-dilution curve (as at the arrow in Chart 2, curve for liver at pH 6.5) and to several of the higher dilutions of the supernatant fluid producing complete hemolysis in nearly the same time.

TABLE 1

TIMES FOR COMPLETE HEMOLYSIS BY LYSINS FROM LUNG AND TUMOR

	Dilution of supernatant fluid						
Tissue	1	2	4	8	16	32	64
Lung	20	25	50	180	480		
Tumor	60	50	30	20	20	40	170

The conclusion that the hemolysins in the supernatant fluids of the pre-incubated homogenates of normal tissue and of tumor are the same, differing only in their concentration, can be further tested by placing both fluids on paper strips in the Durrum electrophoresis apparatus (2), separating the proteins and the lytic components insofar as they can be separated by this method, and afterward identifying their position on the strips (10). At pH 5.6, the supernatant fluid derived from the mouse mammary tumor gives a more complex protein pattern than that derived from mouse lung; only one protein component, with an isoelectric point above pH 5.6, is found in the case of the latter, whereas the former shows at least three components, one with an isoelectric point below pH 5.6. When the lysins are extracted from the strips, they are found to be poorly associated with the proteins and to be substantially the same in the fluids derived from the homogenates of both lung and tumor. There are two lytic components separable at pH 5.6; a less active one is charged negatively, and a more active one is charged positively (Chart 3). The former may be a lysolecithin-like substance with its isoelectric point in the neighborhood of pH 4, and the latter may be a soaplike substance (see below). The only difference observed between the supernatant fluids of pre-incubated tumor homogenates and those of pre-incubated homogenates of normal mouse tissue is that about 4 times as much hemolytic material can be extracted from the strips when the fluid is derived from tumor homogenate. This difference, however, seems to be only a quantitative one, i.e., to reflect a concentration difference only.

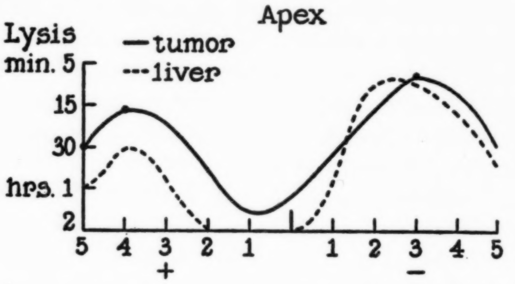

CHART 3.—Lytic components obtained by paper strip electrophoresis at pH 5.6 of supernatant fluids of pre-incubated extracts of mouse liver and mouse breast tumor. No significant difference between them, other than one which can be attributed to concentration, is seen.

LYTIC SUBSTANCES EXTRACTED WITH ORGANIC SOLVENTS

Extraction of minces or homogenates of normal mouse tissue or mouse mammary tumor yields at least two varieties of hemolytic substance, one soluble in alcohol and ether, and the other soluble in alcohol but insoluble in cold ether. The first variety has the properties of fatty acids or soaps and can be referred to as lysolecithin-like (10).

When placed on strips in the Durrum electrophoresis apparatus, both the soaplike and the lysolecithin-like material separate at pH 5.6 into two hemolytic components (Chart 4). The components obtained from extracts of tumors occupy essentially the same positions as those obtained from extracts of liver or lung. Separation at pH 7.6 yields essentially the same result, which again can be interpreted as showing that essentially the same hemolysins are obtained from tumor and from normal tissue by extraction with organic solvents, any difference in activity being due to concentration differences.

It is a tempting hypothesis that the positively charged lytic component of both normal tissue and of tumor is a lysolecithin-like substance which has undergone oxidative changes (11), with the result that its isolectric point is about pH 4. If so, the material separated as lysolecithin-like is not a single substance, for a negatively charged lytic component is present as well. It is also tempting to think of the negatively charged component at pH 5.6–7.6 as a soap, but, if so, it is not a single substance either. These results may be due to the method's being unable to produce complete separation, perhaps because of the well known tendency of lipids and fatty acids to dissolve and to precipitate anomalously in the presence of one another.

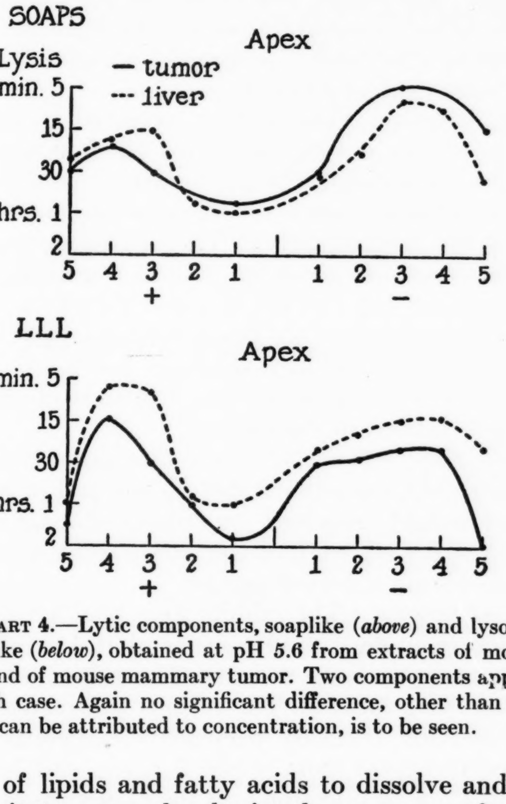

CHART 4.—Lytic components, soaplike (*above*) and lysolecithin-like (*below*), obtained at pH 5.6 from extracts of mouse liver and of mouse mammary tumor. Two components appear in each case. Again no significant difference, other than one which can be attributed to concentration, is to be seen.

SUMMARY

1. No difference, except one which can be attributed to a difference of concentration, has been found between the hemolytic material which can be extracted with saline from the spontaneous mammary carcinoma of the C3H mouse and the hemolytic material which can be extracted with saline from mouse lung and kidney. The time-dilution curves for the lysins from tumor and from normal tissue are atypical and show zones; hemolytic material from both sources hemolyze the red cells of mouse, man, rabbit, and sheep, in that order of increasing resistance, but are not species-specific; lysins from both sources are equally unstable on standing, and both produce agglutination of rabbit, human, and mouse red cells when used in the proper concentration. It is a regular finding, however, that the extracts prepared from the tumors are more hemolytic than those prepared from lung and kidney.

2. The lytic material found in pre-incubated homogenates of the C3H mouse tumor and that found in pre-incubated homogenates of mouse lung are similar; the tumor homogenates, however, usually contain a greater concentration of lysin. The supernatant fluids from both lung and tumor homogenates contain at least two electrophoretically separable hemolytic components, poorly associated with the proteins; one has an isoelectric point below pH 5.6, and the other is still positively charged at pH 8.0.

3. The lytic materials which can be extracted with organic solvents from the C3H tumor have not been found to differ in any respect, except that of concentration, from those extracted from mouse lung. Both soaplike and lysolecithin-like substances can be extracted from both sources, and each contains two electrophoretically separable components.

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The Effect of Prior Injections of Tissue Antiserums on the Survival of Cancer Homoiografts in Mice*

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It had been previously noted (2) that prior injections of lyophilized normal and cancerous mouse tissues resulted either in break-down of host resistance, as evidenced by positive takes of tumor grafts, or in enhanced host resistance, evidenced by the failure of tumor takes. Whether enhancement or inhibition occurred was related to the dose of lyophilized tissue injected (1).

In investigations dealing with wound healing, it was found that the process of healing could be accelerated or retarded by dose gradation of tissue antiserum injections, administered immediately after wounding (3, 4).

The investigations reported here are directed to the question of whether the observed experimental alterations in normal tissue growth (wound healing) and cancerous tissue growth (tumor homoiografts) are under the control of common host mechanisms. To this end, a study was made of the effects of prior injections of tissue antiserums on the growth of cancer homoiografts in mice.

MATERIALS AND METHODS

The host strain of mice used was C57 black/6Ks. These were divided equally according to sex and were 2–3 months old at the start of the experiments. The transplantable tumors used were Sarcoma I and 15091a, both indigenous to the inbred A strain of mice. Sarcoma I is composed of dense sheets of spindle-shaped polyhedral cells. Tumor 15091a is an anaplastic carcinoma, predominantly spindle-celled. Both tumors grow rapidly in 100 per cent of strain A mice. Occasionally, they grow progressively in a C57 black mouse (about 1 in 300).

Antiserums to tissues from strain A mice were produced in rabbits and in C57 black/6Ks mice.

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Antigens.—Fresh tissue homogenates of spleen, kidney, and tumor 15091a in 0.85 per cent saline were mixed in equal proportion for injection. The antigens were freshly prepared for each injection.

Immunization protocol.—

1. Rabbits: Three rabbits received a total of six injections at 4-day intervals. The first injection was intraperitoneal. In subsequent injections the antigen homogenates were divided into two halves by light centrifugation. The sediment was injected intraperitoneally in the morning, in amounts of 1.0, 1.5, 2.0, 2.0, and 2.0 ml. in order, in each of the five injections; 0.5 ml. of the supernate was injected intravenously 4–6 hours later. This procedure lessened the incidence of allergic manifestations in the rabbits.

2. Mice: Fifty C57 black/6Ks mice, about 3 months old, equally divided by sex and of equivalent weight, received six intraperitoneal injections of 0.5 ml. each of the whole tissue homogenates during a 2-week period.

Antiserums.—The rabbits were bled by cardiac puncture, under intravenous nembutal anesthesia (30 mg/kg of body weight). The mice were bled by tail vein incision. The serums were separated, titrated for antibody presence by agglutination of erythrocytes from strain A mice, and lyophilized. Prior to injection into the experimental animals, the antiserums were reconstituted with sterile double-distilled water.

Hemagglutinin titers for the rabbit antiserums were as follows: rabbit No. 1, 1:5120; rabbit No. 2, 1:128; rabbit No. 3, 1:512. The titer for the antiserum produced in the C57 black/6Ks mice was 1:256.

Experimental procedure.—The host mice (C57 black/6Ks) received five intraperitoneal injections of 0.5 ml. each of the reconstituted antiserums over a $2\frac{1}{2}$-week period. Live tumor was inoculated subcutaneously in the suprascapular region by trocar, under aseptic conditions, 7 days after the last injection of antiserum. The course of subsequent growth of the grafts was followed twice weekly by palpation for 1 month, and weekly

TABLE 1

PROTOCOL OF INJECTIONS OF ANTISERUMS INTO C57 BLACK/6Ks MICE*

Mouse group no.	Type of substance injected	Amount per injection (mg. dry wt.)†	Total amt. injected per mouse (mg.)	Tumor graft
Experiment 1:				
1	Rabbit immune serum‡	10	50	Tumor 15091a
2	Rabbit normal serum‡	10	50	“ “
3	Distilled water	(0.5 ml.)	(2.5 ml.)	“ “
4	Uninjected			“ “
5	Lyophilized tumor 15091a	10	50	“ “
6	Rabbit immune serum‡	20	100	Sarcoma I
7	Rabbit normal serum‡	20	100	“
8	Lyophilized Sarcoma I	5	25	“
Experiment 2:				
1	Mouse immune serum‡§	10	50	Sarcoma I
2	“ “ “	20	100	“
3	Mouse normal serum‡	10	50	“
4	“ “ “	20	100	“
5	Uninjected			“

* Live tumor was inoculated subcutaneously 1 week after the last injection of antiserum. The tumor graft used is shown in the last column.

† Volume for each injection 0.5 ml.; 5 injections over 2½-week period.

‡ Immune serum was produced in rabbits or mice, as indicated, to mixed tissue antigens of strain A mouse spleen, kidney, and tumor 15091a. Normal serum from same rabbits or mice before they were immunized.

§ Immune serum produced in C57 black/6Ks mice.

TABLE 2

THE EFFECT OF PRIOR INJECTIONS OF RABBIT AND MOUSE IMMUNE SERUMS AGAINST STRAIN A MOUSE TISSUES ON THE GROWTH OF HOMOIOGRAFTS OF TUMOR 15091a AND SARCOMA I IN C57 BLACK/6Ks MICE*

Substance injected	Total amt. per mouse (mg. dry wt.)	Tumor graft used	C57 black/6Ks hosts dying with tumors†	Growth of graft‡
Experiment 1:				
Immune serum from:				
Rabbit No. 2§	50	15091a	0/30	−
“ No. 3§	“	“	1/27	+ (1)
Normal serum from:				
Rabbit No. 2	“	“	0/19	−
“ No. 3	“	“	0/20	−
Lyophilized 15091a	“	“	10/11	
Distilled water	(2.5 ml.)	“	0/10	
Immune serum from:				
Rabbit No. 1§	100	Sarcoma I	1/19	+ (9)
“ No. 2§	“	“	0/11	+ (1)
Normal serum from:				
Rabbit No. 1	“	“	0/19	−
“ No. 2	“	“	0/11	−
Lyophilized Sarcoma I	25	“	6/10	
Control (nothing)		“	0/12	−
Experiment 2:				
C57 black/6Ks immune serum§	50	Sarcoma I	1/30	+ (19)#
“ “ “ “	100	“	2/8	+ (6)#
C57 black/6Ks normal serum	50	“	0/30	+ (1)
“ “ “ “	100	“	0/8	−
Lyophilized Sarcoma I	50	“	10/12	
Control (nothing)		“	0/15	−

* Live tumor was inoculated subcutaneously 1 week after the last injection. Tumors were simultaneously grafted in strain A mice as tumor viability controls; 100 per cent died with tumors.

† Numerators indicate numbers dying with tumors; denominators the total animals in the group.

‡ + indicates growth of tumor, followed by complete regression; − indicates no growth of the graft. Number in parentheses gives number of + animals.

§ The hemagglutinin titers of the immune serums were: rabbit No. 1, 1:5120; rabbit No. 2, 1:128; rabbit No. 3, 1:512; mouse immune serum, 1:256.

The size of these tumors before regression was comparable to those found in the strain A control animals at death.

thereafter. Negative animals were kept for a minimum of 2 months before being classified as "no-take." Animals with tumors were followed until death.

RESULTS

Table 1 gives the experimental protocol. The findings are given in Table 2.

The data presented in Table 2 clearly show that prior injections of immune antiserums to strain A mouse tissues, whether produced in rabbits or mice, led to partial or complete breakdown of resistance to the tumor homoiografts in a significant number of the C57 black/6Ks hosts. This is shown by the several cases of successful "takes," leading to death of the host, and the large size to which the grafts grew before regressing in a number of the experimental animals. Growth was particularly marked in the animals that had been injected with the immune serum produced in mice. In the control hosts the grafts grew not at all, or grew slightly and regressed rapidly.

The data also indicate that there may be a relationship between the relative strengths of the rabbit antiserums (as indicated by their hemagglutinin titers) and the magnitudes of their effect, as measured by the altered reactions of the hosts to the homoiografts. The decreasing order of effectiveness of the three rabbit immune serums is the same as the relative order of their hemagglutinin titers.

It is of interest that the mouse immune serum, with a relatively low hemagglutinin titer, had a much more marked effect than the rabbit serums. The reason for this is not known.

In the case of the mouse antiserum, the magnitude of the effect appears to parallel the amount injected, in terms of dry weight. If we consider that the hemagglutinin titer is a measure of the amount of antiserum present, then these findings are in accord with the larger effect noted with the higher titer rabbit antiserums.

The effects of the antiserums on host-graft relationships parallel our previously reported results on the effects of lyophilized mouse tissues on tumor homoiografts (2).

SUMMARY

Antiserums were produced in rabbits and C57 black/6Ks mice to mixed antigens of spleen, kidney, and tumor from strain A mice. Prior injections of the antiserums into C57 black/6Ks mice led to marked growth, and some takes, of two strain A tumors. These tumors normally regress in nearly 100 per cent of C57 black/6Ks mice.

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Serum Tributyrinase Levels in Mice of the C57, C3H, and A Strains*

JULES TUBA

WITH THE TECHNICAL ASSISTANCE OF IRMGAARD JESKE

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A series of three papers by Khanolkar and Chitre (1, 3, 4) dealt with esterase studies in a cancer-resistant strain of mouse, C57, and in two cancer-susceptible strains, C3H and A. These authors showed that the hydrolysis of ethyl butyrate by mouse serum is twice as great in the cancer-susceptible animals as in the cancer-resistant group. There was no difference found in enzyme activity associated with the sex or age of the animals.

Studies in this laboratory on serum alkaline phosphatase (7), serum tributyrinase (10), and mammary cytochrome system of rats (8) have indicated that the sex and age of the animals may have significant effects on the levels of the enzymes. Our studies on the oxygen uptake of rat mammary tissue have demonstrated significant differences between breeder and virgin females in this regard. Unpublished findings with mice have shown us that age, sex, and previous pregnancy can affect the levels of certain enzymes. Consequently, it was decided to examine the effect of these factors on the serum tributyrinase concentrations of the strains of mice used by Khanolkar and Chitre. Our decision was strengthened by the fact that the simple titrimetric micromethod of Tuba and Hoare (9) was readily adaptable to investigations with mouse serum.

EXPERIMENTAL

Mice of various representative ages were housed in groups in metal cages and given Purina Fox Checkers and water ad libitum. Males and virgin females were segregated from the beginning of the experiment. Breeder females were segregated at least 4 weeks after the last litter was weaned, to eliminate any possible effect of lactation on the levels of the serum enzyme. There were 205 C3H, 104 C57, and 92 A mice used in the investigation.

For tributyrinase studies the animals were killed by decapitation, and blood was collected in 2-ml. conical centrifuge tubes. Serum was removed as soon as possible and stored at 4° C. until lipase determinations were done. This was never delayed for more than 3 days, although the enzyme was found to be stable for several days. For example, a sample of serum which showed an initial activity of 930 units was found to have an activity of 910 units/100 ml 4 days later, and this is within the limits of experimental error.

Micro-Kjeldahl tubes, which have bulbs of about 5 ml. in capacity, are used for the tributyrinase estimations. Into one of these tubes are pipetted with mixing: 0.05 ml. serum, 0.2 ml. water, and 1.0 ml. veronal buffer of pH 8.2. The mixture is warmed to 37° C., and then 0.02 ml. tributyrin, previously warmed to 37° C., is added with vigorous shaking of the contents of the experimental tube, in order to disperse the substrate. The contents of the tube are then agitated on a Warburg bath at the rate of 120 swings per minute, which is sufficient to maintain the substrate in a state of fine dispersion. It was found that under the above experimental conditions, which are optimal for studies of mouse tributyrinase activity, a hydrolytic period of 1 hour released sufficient butyric acid for accurate titration. Kinetic studies showed that enzyme action is proportional to time during this period and that activity varies directly with enzyme concentration for the amount of serum used.

After 1 hour, enzyme action in the experimental tube is terminated by the addition of 3 ml. of 95 per cent ethyl alcohol. The mixture is centrifuged, and the supernatant liquid is poured into a 50-ml. Erlenmeyer flask, which is immediately stoppered to prevent absorption of carbon dioxide. The contents of the flask are titrated with 0.025 N sodium hydroxide, with the use of phenolphthalein as an indicator. A control tube is used in which

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the serum is boiled before the addition of buffer and substrate.

The difference between titration values for the experimental and control tubes is a measure of the activity of the enzyme. Tributyrinase activity of mouse serum in units is equivalent to the number of ml. of 0.025 N sodium hydroxide required to neutralize the amount of butyric acid which is set free by the enzyme contained in 100 ml. serum under the above conditions. In replicate determinations the average deviation from the mean value was 2–3 per cent.

RESULTS

Each of the three strains was divided into a group of young animals (12–20 weeks), a middle-aged group (30–40 weeks), and an old group (50–60 weeks). The animals were also grouped as males, breeder females, and virgin females. In Table 1 the ranges of tributyrinase levels and means are given for each group of mice. The standard errors of the means are given for the total males, breeders, and virgins of each strain, and they are, in every case, less than 5 per cent of the mean value.

The statistical analysis of the tributyrinase means given in Table 1 is presented in Table 2. The data for the various groups have been compared for differences which might be due to age, sex, or strain. Those comparisons which show statistically significant differences are included in the table. In each case the t value is given, and levels of significance are indicated by values of P.

Probability of 0.05 or less is significant, while a probability of 0.01 is highly significant.

DISCUSSION

It can be seen in Table 2 that age has a significant effect on serum tributyrinase levels in some groups. However, it cannot be said that any definite age pattern exists which may be associated with susceptibility or resistance to mammary carcinoma. Breeder females of the three strains show an age effect, which in the case of the C3H group is the reverse of the trend observed in the other two strains. Age effects are found with males and virgins, but these are not common to the three strains.

The effect of sex on serum tributyrinase levels, like that of age, is most pronounced in C3H mice, and males, breeders, and virgins of this strain are all significantly different in one or another of the age groups. Male-breeder differences and male-virgin differences exist in all three strains. Breeder-virgin differences are significant in C3H and C57 mice and are just beyond the range of significance in the A mice. The trend in the A strain would probably be more definite with a larger group of animals. Hormonal influences associated with the sex and previous pregnancy are therefore factors in determining the serum tributyrinase levels of the three strains of mice used in the investigation.

There are highly significant strain differences among the breeders and also among the males. The virgins of the three strains show no significant difference in enzyme level. We find, as do Khanolkar and Chitre with their esterase, that mice of the three strains, undifferentiated as to age, sex, and previous pregnancy, have significantly different levels of serum tributyrinase. The mean values, with standard errors of the means, for C57, A, and C3H mice, respectively, are 932 ± 12, 1045 ± 18, and 900 ± 8 units/100 ml of serum tributyrinase. There is no relationship between these levels and the degree of mammary cancer incidence; in fact, the mean for the C3H strain is less than that for the C57 animals. Shimkin, Greenstein, and Andervont (6) found that there is no correlation between the degree of hydrolysis of methyl butyrate by mouse serum and the occurrence of murine mammary tumors.

There are a number of factors which could account for the points of disagreement which exist between our results and those of Khanolkar and Chitre. The number of animals used has, of course, an important bearing on the statistical evaluation of results. It was noted above that the breeder-virgin difference in the A strain is just below the level of significance, probably because the numbers of animals are smaller than in the other groups. Variations in technic undoubtedly account for some of the differences between the two sets of results. It is our experience that the use of boiled serum in the control tube gives a more accurate measurement of the amount of hydrolysis which occurs in the experimental tube, since the buffering capacities of various sera are not always the same. A shorter hydrolytic period is usually preferable. Since the substrate was apparently not emulsified in the esterase determinations of Khanolkar and Chitre, it is possible that saturation of the enzyme was not always achieved.

We have estimated the extent of hydrolysis of ethyl butyrate by the sera of four 20-week-old male mice of each of the three strains. The method of Tuba and Hoare (9) employs veronal buffers, and, by the use of their technic, optimum hydrolysis was obtained at pH 7.8. The values for 20-week-old male C3H, C57, and A mice are respectively 390 ± 10, 417 ± 12, and 387 ± 18. These results, like those of Shimkin *et al.* (6),

TABLE 1

Serum Tributyrinase Levels

Males				Breeder females				Virgin females			
		Tributyrinase (units/100 ml)				Tributyrinase (units/100 ml)				Tributyrinase (units/100 ml)	
Age (weeks)	No. of mice	Range	Mean	Age (weeks)	No. of mice	Range	Mean	Age (weeks)	No. of mice	Range	Mean
C3H Strain											
12–20	8	740– 930	848					12–20	18	750–1,040	935
30–40	66	710–1,000	830	30–40	47	750–1,160	939	30–40	31	710–1,040	888
50–60	17	800–1,150	973	50–60	14	910–1,220	1,088	50–60	4	750– 950	807
Total	91	710–1,150	858±10*	Total	61	750–1,220	1,004±16*	Total	53	710–1,040	889±15*
A Strain											
12–20	10	810–1,440	1,149					12–20	12	810–1,280	971
30–40	23	820–1,370	1,104	30–40	16	810–1,320	1,121				
50–60	20	810–1,240	1,040	50–60	5	690–1,010	840	50–60	6	840–1,100	906
Total	53	1,040–1,149	1,073±23*	Total	21	690–1,320	1,054±44*	Total	18	810–1,280	952±36*
C57 Strain											
12–20	12	720–1,110	980					12–20	15	710– 970	852
30–40	13	710–1,170	1,001	30–40	6	850–1,000	958	30–40	10	790–1,050	919
50–60	14	700–1,100	947	50–60	14	760–1,000	869	50–60	10	870–1,100	969
Total	39	700–1,170	976±22*	Total	20	760–1,000	896±21*	Total	35	710–1,100	904±16*

* Standard error of the mean.

TABLE 2

Statistical Analysis of Data of Table 1

Strain	Groups compared	Value of t	Value of P
Age differences			
C3H	12–20-week males vs. 50–60-week males	3.33	<0.01
C3H	30–40-week breeders vs. 50–60-week breeders	5.01	<0.01
C3H	12–20-week virgins vs. 50–60-week virgins	2.53	0.01–0.02
A	30–40-week breeders vs. 50–60-week breeders	3.56	<0.01
C57	30–40-week breeders vs. 50–60-week breeders	2.88	<0.01
C57	12–20-week virgins vs. 50–60-week virgins	2.64	0.01–0.02
Sex differences			
C3H	30–40-week males vs. 30–40-week breeders	6.02	<0.01
C3H	50–60-week males vs. 50–60-week breeders	3.09	<0.01
C3H	12–20-week males vs. 12–20-week virgins	2.54	0.01–0.02
C3H	50–60-week males vs. 50–60-week virgins	3.01	<0.01
C3H	50–60-week breeders vs. 50–60-week virgins	5.16	<0.01
C3H	All males vs. all breeders	7.84	<0.01
C3H	All breeders vs. all virgins	5.45	<0.01
A	50–60-week males vs. 50–60-week breeders	2.79	0.01–0.02
A	50–60-week males vs. 50–60-week virgins	2.11	0.02–0.05
A	All males vs. all virgins	2.88	<0.01
C57	50–60-week breeders vs. 50–60-week virgins	2.68	0.01–0.02
C57	12–20-week males vs. 12–20-week virgins	2.81	<0.01
C57	All males vs. all breeders	2.70	<0.01
C57	All males vs. all virgins	2.68	<0.01
Strain differences			
C57 and C3H	Total breeders vs. total breeders	4.26	<0.01
C57 and A	Total breeders vs. total breeders	3.19	<0.01
C57 and C3H	Total males vs. total males	4.89	<0.01
C57 and A	Total males vs. total males	3.09	<0.01
C57 and C3H	Total mice vs. total mice	2.19	0.01–0.02
C57 and A	Total mice vs. total mice	5.06	<0.01

show no correlation between enzyme activity and susceptibility to cancer.

Our findings are negative in the sense that we find no distinction in serum tributyrinase levels which can be associated with the incidence of murine mammary cancer. On the other hand, the results are in keeping with our observations of other enzymes in rats and mice: that is, age, sex, and previous pregnancy are factors which may affect enzyme activity and which should be taken into account. Morrow, Carroll, and Greenspan (5) have found that sex and age are factors in determining kidney glucuronidase levels in inbred mice. Harris and Cohen (2) report a pronounced influence of ovarian hormones on the activity of various enzymes, including esterase, in the tissues of Wistar strain female mice.

SUMMARY

1. Serum tributyrinase levels have been determined for mice of the C3H, A, and C57 strains.

2. Statistically significant effects on enzyme activity are associated with age, sex, previous pregnancy, and strain.

3. There is no evidence that the serum tributyrinase activity is related to susceptibility or resistance to cancer.

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The Effects of Freezing, Storage, and Thawing upon the Transplantability of Mouse Leukemic Cells*†

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The need for a dependable method for the storage and preservation of viable tumor tissues has long been of concern to investigators in experimental oncology. This problem is of particular interest in the study of mouse leukemia, where the maintenance of any given line of leukemia depends upon continuous serial transfer through successive generations of inbred mice of known genetic constitution—a procedure that is costly in time and materials. In attempts to solve this problem, low temperatures for the preservation of cells have been employed with varying and conflicting reports of success. Commonly, these differences can be attributed to the inherent complexity of any of the variety of methods available for the preparation and preservation of cells. Thus, of particular concern and interest are the procedures for the preparation of tissues prior to freezing, the ingredients and temperature of the cooling bath, the method and time allotted for freezing and storage, and the method and time required for thawing frozen material in preparation for its injection into susceptible animals. Each of these factors has been shown, at one time or another, to be of importance.

A recognition of the many deficiencies in existing methods and information led to the present series of investigative studies on the effects of freezing and thawing upon the transplantability of mouse leukemic cells. The present report contributes to an understanding of the effects of alterations in temperature and in the duration of storage (*a*) by making it known that the loss of cellular viability from subjecting leukemic cells to abrupt freezing, storage, and rapid thawing is limited to 72 hours for three lines of leukemia and (*b*) by demonstrating, in confirmation of earlier studies, a negligible detrimental effect upon leukemic cells when the processes of freezing and thawing are carried out slowly.

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† A preliminary report of this work was presented at the meeting of the American Association for Cancer Research at Cleveland, April 27–29, 1951 (Cancer Research, 11:249, 1951).

‡ The material contained in this paper appeared in part in a thesis submitted in partial fulfillment of the requirements for the degree of Master of Science, and it will appear in part in a thesis to be submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of Minnesota.

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MATERIALS AND METHODS

Leukemic cells.—Leukemic cells representative of two lines of lymphoid leukemia, 876 and 926, and one line of myeloid leukemia, 765, were employed. These lines of leukemia originated spontaneously in strain F inbred mice in the laboratory of one of the authors (A. K.), where they have been maintained by serial transmission through many generations.

Animal hosts.—The treated tissues were tested for viability by injection into F_1 hybrid mice which had been derived from a cross of an inbred F male with a female of any other strain.

Leukemic tissue supply.—A standard inoculum of 100,000 cells for the F_1 host mice on intraperitoneal injection insured the constant onset of leukemia and a satisfactory volume of leukemic spleen tissue.

Preparation of the 50 per cent leukemic splenic suspension for experimentation.—Leukemic donor mice were killed by cervical dislocation, and their bodies were dipped in 70 per cent alcohol. The spleens were removed, pooled, and mashed with the aid of a screw-type press with a 22-gauge disc pore size. The tissue mash was weighed, an equal volume of 5.3 per cent glucose was added (9), and the materials after mixture were distributed in 1.4-ml. portions in ampules. These containers were labeled, sealed with a hot-flame burner, and cooled in the refrigerator (4° C.) for 5 minutes before freezing.

Procedures for freezing and thawing.—In the experiments to be described the terms "slow freeze," "fast freeze," "slow thaw," and "fast thaw" will be employed for ease of description. Slow freeze (SF) signifies that the content of an ampule was frozen slowly by immersion in a methyl cellosolve[1] bath, the temperature of which was decreased from 4° C. to −76° C. by the addition of crushed dry ice over a period of 20 minutes. A fast freeze (FF) was effected by the sudden immersion of an ampule into a methyl cellosolve and dry ice bath at −76° C., where it was kept for 3 minutes. All materials which had been prepared by these freezing procedures were kept in a dry ice storage chest, where the temperature was maintained at approximately −76° C. In a slow thaw (ST), the ampules from a −76° C. dry ice storage chest were kept successively at 4° C. for 10 minutes, at room temperature for 10 minutes, and between the palms of the hands where thawing was completed by rotating the ampule. For a fast thaw (FT) the ampules were removed from the dry ice storage chest and quickly immersed in a beaker of water at 37° C. The thawing process was completed in less than 1 minute.

Mouse inoculation.—The intraperitoneal route was chosen as the most effective method for the introduction of material into mice in tests for the viability of leukemic cells. A standard inoculum for the test injection of fresh and frozen materials consisted of 0.1 ml. of 50 per cent splenic mash.

Evaluation of cell viability.—All animals which had been inoculated with fresh or treated material were carefully observed to determine the development of leukemia. Each animal was palpated daily for evidence of an enlargement of its spleen, or lymph nodes, or of tumor growth; a positive "take" was recorded the first day that evidence of any enlargement was observed. In the reporting of the results of the animal inoculations (T/I), the numerator, T, represents the number of positive transplantations and the denominator, I, the total number of mice that were inoculated. Each mouse recorded as "positive" was kept under observation until death, when examination at autopsy was performed to confirm the presence of leukemia. All the animals that failed to give evidence of successful transplantation were observed for at least 100 days.

EXPERIMENTAL

Two series of experiments were carried out to determine the effects of storage upon the transplantability of strain F mouse leukemic cells. These two series differed principally in the methods by which leukemic cells were treated in preparation for storage.

[1] Methyl "cellosolve" solvent (ethylene glycol monomethyl there), a product of Carbide and Carbon Chemicals Division.

Series A: Slow Freeze-Slow Thaw

Even though procedures utilizing changes in temperature by freezing slowly and thawing slowly for preserving tumor tissues are reported (2–5, 7, 8, 13, 14, 16, 17) to produce a high yield of transplantable material, the experiments in Series A were designed to test the effects of storage on the transplantability of leukemic cells after their preparation by freezing slowly, storage at −76° C. for definite periods of time, and slowly thawing to room temperature prior to inoculation into susceptible mice. This procedure is referred to as "slow freeze-slow thaw" (SF-ST).

TABLE 1

The Effects of Storage upon the Incubation Period of Mouse Myeloid Leukemia, Line 765,* Prepared by the "Slow Freeze-Slow Thaw" Technic

Period of observation in days	Period of storage in hours: 12	24	36	48	60	72	Fresh tissue control
0	0/5†	0/5	0/5	0/5	0/5	0/5	0/5
7							1/5
8							2/5
12							3/5
13			1/5				
14	2/5	1/5					4/5
15					3/5	2/5	5/5
16			2/5	3/5			
17	4/5						
22						4/5	
23			5/5	4/5			
31		2/5					
36					4/5	5/5	
38		4/5					
41				5/5			
50		5/5					
62					5/5		
100	4/5	5/5	5/5	5/5	5/5	5/5	5/5

* Transfer generation 33.

† Denominator signifies number of recipient mice; numerator, the mice that developed leukemia.

Experiment 1.—Ampules containing a splenic mash of mouse myeloid leukemia, line 765, transfer generation 33, in 50 per cent suspension, were frozen slowly in methyl cellosolve and dry ice and stored at −76° C. At 12-hour intervals single ampules of material were removed and slowly thawed before the contents were employed for inoculation into F_1 hybrid mice.

The results of this experiment are presented in Table 1.

The results of Experiment 1 show (*a*) that the incubation period for the onset of leukemia was greater in the mice that had received the SF-ST-treated material than it was in the mice which had

been inoculated with freshly prepared, unfrozen material; (*b*) that freezing and thawing slowly had little or no effect upon the viability of the cells; (*c*) that the duration of storage did not produce any significant effect upon the number of positive transplants obtained; and (*d*) that the fresh, unfrozen material produced leukemia in 100 per cent of the animals of the control group.

TABLE 2

THE EFFECTS OF STORAGE UPON THE INCUBATION PERIOD OF MOUSE LYMPHOID LEUKEMIA, LINE 876,* PREPARED BY THE "SLOW FREEZE-SLOW THAW" TECHNIC

PERIOD OF OBSERVATION IN DAYS	72-HOUR STORED MATERIAL	FRESH TISSUE CONTROL
0	0/10†	0/10
8		6/10
9		8/10
11		10/10
12	3/10	
13	4/10	
18	5/10	
20	6/10	
21	8/10	
100	8/10	10/10

* Transfer generations 42 and 43.

† Denominator signifies number of recipient mice; numerator, the mice that developed leukemia.

The results obtained with frozen tissue mash of line 765 made it desirable for confirmation to test the effect of storage upon the transplantability of slowly frozen and thawed splenic tissue of mouse lymphoid leukemia, line 876. Since it had been observed in Experiment 1 that the SF-ST method of preparation with storage for from 12 to 72 hours did not produce a significant difference in the number of positive transplantations, Experiment 2 employed only SF–ST-treated material which had been stored for 72 hours.

Experiment 2.—Ampules of mouse leukemic tissue, line 876, transfer generations 42 and 43, a 50 per cent leukemic splenic suspension, were prepared and treated by SF-ST with 72-hour storage prior to inoculation into F_1 hybrid animals. The results of this experiment are presented in Table 2.

It is evident from the results of Experiment 2 (*a*) that material which had been treated with the SF-ST method required a longer period of incubation for the development of leukemia than was needed for the fresh tissue controls; (*b*) that freezing and thawing slowly had some effect on the viability of the cells, in that only 80 per cent of the recipients gave evidence for growth on transfer of tissues which had been stored for 72 hours; and (*c*) that the freshly prepared tissue suspension was capable of producing leukemia in all mice of the control group. Thus, these results confirmed satisfactorily the findings that had been obtained in Experiment 1.

In Experiment 3 the purpose was to obtain additional confirmatory evidence on the value of the SF-ST treatment in the preservation of viability of mouse leukemic cells.

Experiment 3.—Splenic tissue from line 926, transfer generations 53 and 69, mouse lymphoid leukemia was prepared and treated in accordance with the procedure outlined in Experiment 2. The results of this experiment are presented in Table 3.

The results of Experiment 3 show (*a*) that the incubation period required for the development of leukemia with SF–ST-treated material closely approximated that of the control group; (*b*) that slow freezing and thawing produced no effect on the viability of the cells; and (*c*) that all mice inoculated with freshly prepared material developed leukemia.

SERIES B: FAST FREEZE-FAST THAW

The series of experiments was undertaken to determine the effect of storage upon the transplantability of leukemic cells that were made ready for storage by a process which consisted of abrupt freezing, maintenance in storage at a known temperature for graded, predetermined intervals of time, and rapid restoration to room temperature. This sequence, for descriptive convenience, is referred to as "fast freeze-fast thaw" (FF-FT).

TABLE 3

THE EFFECTS OF STORAGE UPON THE INCUBATION PERIOD OF MOUSE LYMPHOID LEUKEMIA, LINE 926,* PREPARED BY THE "SLOW FREEZE-SLOW THAW" TECHNIC

PERIOD OF OBSERVATION IN DAYS	72-HOUR STORED MATERIAL	FRESH TISSUE CONTROL
0	0/10†	0/10
8		1/10
9	1/10	5/10
10		7/10
11		9/10
13	5/10	
14	8/10	10/10
16	10/10	
100	10/10	10/10

* Transfer generations 53 and 69.

† Denominator signifies number of recipient mice; numerator, the mice that developed leukemia.

Experiment 4.—Ampules containing a 50 per cent suspension of splenic tissue representative of mouse myeloid leukemia, transfer generation 30, line 765, were frozen rapidly in a methyl cel-

losolve and dry ice bath and stored at −76° C. At successive 12-hour intervals thereafter, a single ampule was removed, quickly thawed, and injected into F_1 hybrid animals. The results of this experiment are presented in Table 4.

The results of Experiment 4 made it known (*a*) that the incubation period for the production of leukemia with FF-FT material exceeded, by almost twice, the time interval that was required for the development of leukemia in mice that had been inoculated with unfrozen, freshly prepared material; (*b*) that rapid freezing and thawing resulted in a total loss of viability when the material was tested during the first 36 hours, partial loss in the period from 48 to 60 hours, and positive transfer at 72 hours; (*c*) that the number of positive transfers was in a linear relationship to the duration of storage; and (*d*) that freshly prepared, unfrozen material produced leukemia in all animals of the control group.

TABLE 4

THE EFFECTS OF STORAGE UPON THE INCUBATION PERIOD OF MOUSE MYELOID LEUKEMIA, LINE 765,* PREPARED BY THE "FAST FREEZE-FAST THAW" TECHNIC

PERIOD OF OBSERVATION IN DAYS	PERIOD OF STORAGE IN HOURS 12	24	36	48	60	72	FRESH TISSUE CONTROL
0	0/10†	0/10	0/10	0/10	0/10	0/10	0/10
7							2/10
8							4/10
12							6/10
14							8/10
15							10/10
27						1/9	
29						4/9	
45					1/10	5/9	
46				1/10			
48						6/9	
55					2/10		
100	0/10	0/10	0/10	1/10	2/10	6/9	10/10

* Transfer generation 30.

† Denominator signifies number of recipient mice; numerator, the mice that developed leukemia.

From the results obtained with frozen tissue mash of line 765, special experiments were designed to test the effect of storage upon the transplantability of frozen and thawed lymphoid leukemic tissue of line 876. It had been noted that material which had been stored for 36 hours did not produce a transplantable leukemia, while other samples of material which had been stored for 72 hours were able to produce a great number of positive takes. As a result of these findings, two storage periods, 36 and 72 hours, were used for testing the transplantability of FF–FT-treated material.

Experiment 5.—Ampules containing a 50 per cent leukemic splenic mash representative of mouse lymphoid leukemia, line 876, transfer generations 42 and 43, were rapidly frozen and stored at −76° C. for 36 hours or for 72 hours. These materials were rapidly thawed and inoculated intraperitoneally in F_1 hybrid mice. The results of this experiment are presented in Table 5.

It is evident from the results of Experiment 5 (*a*) that the FF-FT process for treatment resulted in a prolongation of the period of incubation for the development of leukemia over that observed in animals that had been given fresh tissue; (*b*) that FF-FT and storage for 36 hours produced a partial loss of viability and that an increase in positive takes was obtained from the injection of the 72-hour preserved material; (*c*) that the number of positive takes from the FF-FT material was directly proportional to the duration of storage; and (*d*) that the freshly prepared, unfrozen tissue suspension was capable of producing leukemia in all mice of the control group.

The purpose of Experiment 6 was to establish by confirmation with the aid of a second lymphoid strain of leukemia, line 926, further evidence of the beneficial effects of storage in combination with the FF-FT technic on the transplantability of leukemic cells.

TABLE 5

THE EFFECTS OF STORAGE UPON THE INCUBATION PERIOD OF MOUSE LYMPHOID LEUKEMIA, LINE 876,* PREPARED BY THE "FAST FREEZE-FAST THAW" TECHNIC

PERIOD OF OBSERVATION IN DAYS	PERIOD OF STORAGE IN HOURS 36	72	FRESH TISSUE CONTROL
0	0/10†	0/10	0/10
8			6/10
9			8/10
11			10/10
12		1/10	
13			
14	1/10		
16		3/10	
17	3/10		
18		6/10	
20			
21			
25		7/10	
100	3/10	7/10	10/10

* Transfer generations 42 and 43.

† Denominator signifies number of recipient mice; numerator, the mice that developed leukemia.

Experiment 6.—Splenic tissues representative of line 926, transfer generations 53 and 69, mouse lymphoid leukemia, were prepared and treated as described in Experiment 5. The results of this experiment are presented in Table 6.

In the results of Experiment 6 it can be seen (*a*) that the incubation period was more prolonged following the transplantation of tissues that had been fast frozen and thawed over that observed in the control group of mice that received fresh tissue; (*b*) that FF-FT produced a high loss of viability in the 36-hour preserved material, in contrast to a slight loss of viability in the 72-hour stored tissue; (*c*) that the number of positive transplants from FF-FT material was in direct proportion to the duration of storage; and (*d*) that leukemia developed in all the mice that had been inoculated with freshly prepared material.

TABLE 6

THE EFFECT OF STORAGE UPON THE INCUBATION PERIOD OF MOUSE LYMPHOID LEUKEMIA, LINE 926,* PREPARED BY THE "FAST FREEZE-FAST THAW" TECHNIC

PERIOD OF OBSERVATION IN DAYS	PERIOD OF STORAGE IN HOURS 36	72	FRESH TISSUE CONTROL
0	0/10†	0/10	0/10
8			1/10
9			5/10
10			7/10
11			9/10
13		1/10	
14		2/10	10/10
16			
17		3/10	
18		5/10	
20		9/10	
21	1/10		
100	1/10	9/10	10/10

* Transfer generations 53 and 69.

† Denominator signifies number of recipient mice; numerator, the mice that developed leukemia.

The results of the experiments that were carried out to learn the effects of storage upon the transplantability of strain F mouse leukemic cells are compiled in Table 7. This table summarizes the differences that were observed when cells which had been prepared by the fast freeze-fast thaw technic, on one hand, were compared to cells prepared by the slow freeze-slow thaw technic.

DISCUSSION

From the results that were presented in this paper it is apparent that the rate and temperature of freezing and the temperature and duration of storage are factors that must be known and controlled in procedures designed to maintain the viability of tumor cells. These studies were undertaken because of the need for accurate information that will make possible the preservation of tumor tissue in anticipation of its transplantation at some future date to susceptible hosts. Three lines, two lymphoid and one myeloid, of strain F leukemia were employed to provide the cells for comparative studies that differed principally in the rate of freezing and thawing. Surprisingly, it was learned for all three lines of leukemia that marked inhibition of the multiplication of leukemic cells occurred when the cells had been frozen abruptly at a temperature of $-76°$ C., kept at that temperature, and, finally, thawed rapidly immediately before transfer to susceptible recipients. For example, following storage, cells of line 765 were tested for viability by transfer to normal recipients. The cells that had been stored (*a*) for periods up to 36 hours did not result in leukemia on injection, (*b*) from 48 to 60 hours produced leukemia in 10 per cent of the recipients, and (*c*) for 72 hours resulted in an incidence of 76 per cent. These results contrast with entirely comparable control experiments that employed slow freezing and slow thawing. These latter studies showed that freezing slowly, storage for from 12 to 72 hours, and slow restoration to room temperature resulted in a satisfactory preservation of cells, as evidenced by leukemia in 94 per cent of the recipients.

The findings herewith reported make understandable the discrepancies in the literature (1, 2, 4, 5, 10, 12, 14, 16) that have resulted from carrying out tests for the viability of tumor cells within 24 hours of rapid freezing. Thus, several investigations (1, 5, 10, 12) yielded results in experiments utilizing myeloid and lymphoid leukemic mouse tissues that were interpreted to indicate destruction of the viability of cells from rapid exposure to temperatures of from $-18°$ C. to $-30°$ C. for a period of 30 minutes. Other studies (2, 7, 8, 13, 14, 16, 17) in which the rates of freezing and thawing were varied report that slowly decreasing the tem-

TABLE 7

SUMMARY ON THE EFFECTS OF STORAGE UPON THE TRANSPLANTABILITY OF STRAIN F MOUSE LEUKEMIC CELLS PREPARED BY THE "SLOW FREEZE-SLOW THAW" AND "FAST FREEZE-FAST THAW" TECHNICS

FREEZING MIXTURE	METHOD OF TREATMENT	PERIOD OF STORAGE IN HOURS	LEUKEMIA FOLLOWING TRANSPLANTATION No. of mice	Per cent leukemic
Methyl cellosolve and dry ice	SF-ST*	12–72	47/50†	94.0
	FF-FT‡	12–60	7/70	10.0
	FF-FT	72	22/29	75.9
Control	Fresh splenic cells only		55/55	100.0

* SF-ST signifies "Slow Freeze-Slow Thaw."

† Denominator signifies number of recipient test mice; numerator, the mice with leukemia.

‡ FF-FT signifies "Fast Freeze-Fast Thaw."

perature of tumor cells in preparation for transplantation resulted in growths in a higher percentage of the recipients than did rapid freezing. For example, Barnes and Furth (2) learned, from studies relating the rate of freezing in dry ice and methyl cellosolve to periods of storage ranging from 30 minutes to 7 hours, that slow freezing and thawing of strain S2 leukemic cell suspensions at either $-30°$ C. or $-70°$ C. resulted in leukemia in 76 per cent of the inoculated animals. They concluded that the duration of exposure was not a determinant of critical importance for the survival of the agent of leukemia. Similarly, in the present investigation the injection of line 765 which had been frozen and thawed slowly produced leukemia in 80 per cent of the cases after storage for 12 hours and in 100 per cent of the cases after 24, 36, 48, 60, and 72 hours of storage. Experiments in confirmation of the above results utilizing materials treated by slowly freezing and thawing and a 72-hour period of storage were successful in producing positive transplants for line 876 in 80 per cent of the recipients and for line 926 in 100 per cent. Since the accumulative experimental findings for the process of freezing and thawing slowly yielded an incidence of 94 per cent, it was concluded that the duration of storage exerted little influence upon the transplantability of treated leukemic cells when the inoculum consisted of cells which had been prepared by freezing and thawing slowly. These results are in agreement with reports of other investigators (2–5, 7, 8, 13, 14, 16, 17) concerning the use of a slow freeze and thaw method of preservation of tumor tissue. On the other hand, the period of storage was shown to have a direct bearing on the transplantability of rapidly frozen and thawed leukemic tissue. Indeed, the duration of storage prior to inoculation determined the success or failure of the transplantation. This effect was first made known by the demonstration for line 765 of a total loss of viability for materials tested during the first 36 hours after treatment, partial to total loss in the period from 36 to 60 hours, and a positive transfer at 72 hours. In contrast to these findings, several investigators (1, 5, 10, 12) obtained results in experiments utilizing myeloid and lymphoid tissues of mice that suggested the destruction of the transmitting agent upon rapid exposure to temperatures of from $-13°$ C. to $-30°$ C. for a period of 30 minutes. Moreover, Barnes and Furth (2) reported that a fast freeze and thaw treatment of strain S2 leukemic cell suspensions rendered inactive, as determined by transplantation, material that had been stored at $-70°$ C. for 30 minutes, while similar samples stored at $-30°$ C. for 30 minutes were capable of producing leukemia in 65 per cent of the recipients. Thus, any differences in results are attributable to the failure to test for viability after 72 hours, or more, of storage at $-70°$ C. Similarly, confirmatory experiments utilizing splenic tissues of mouse lymphoid leukemia, lines 876 and 926, showed (*a*) a noticeable loss of viability when tested after 36 hours' storage and (*b*) a definite capacity to produce leukemia when stored for 72 hours.

The prolongation of the incubation period in the recipients of the slowly frozen material suggested that some cells had been killed during the freezing process. This observation is in agreement with the reports of the investigators (2, 3, 5, 7, 8, 13, 14, 16, 17) who employed slow freezing and thawing as a procedure for the preservation of tumor cells. It is of interest to relate this observation to other studies (2, 6, 10–12, 15) in which it was found (*a*) that the longevity of mice which had been inoculated with fresh leukemic tissue is in inverse relation to the number of cells in the inoculum and (*b*) that life expectancy was prolonged when treated cells were employed. These findings led to the suggestion that cells were destroyed during the process of freezing. Recently, Warner, Gostling, and Thackray (18, 19) reported that Sarcoma 37 tissues which had been either slowly or rapidly frozen resulted, on transplantation, in successful transfer. However, the time interval for growth was slightly longer than that when the corresponding untreated materials had been employed. These investigators were led to conclude from microscopic examination (19) of the growths derived from test materials which had been frozen slowly, frozen rapidly, or kept unfrozen for control purposes that the number of residual tumor cells was roughly proportional to the tumor-producing activity of the material in question. From these results and from the findings reported in this paper, it can be assumed that the increase in the time interval for the onset of leukemia in mice inoculated with treated materials reflects the destruction dur ng the periods of freezing, storage, and/or thawing of cells in numbers sufficient to prolong the incubation period but insufficient to interfere with the success of transplantability.

The inhibition of growth over a period of 72 hours that was observed for the tests employing rapid freezing and thawing suggests the preservation of a labile inhibiting factor, probably enzymatic in nature, that deteriorates during storage and is destroyed by the slow freeze-slow thaw process. Experimental studies are under way to learn the factor or mechanism responsible for the inhibitory action.

SUMMARY

The success of transplantability of three lines of strain F leukemia cells was related to the duration of storage and to the rates of freezing and thawing. Tests for viability by transplantation of the treated leukemic cells to F_1 hybrid mice made it apparent (*a*) that freezing and thawing slowly had little or no effect upon the viability of the cells; (*b*) in contrast to the aforementioned findings, that freezing and thawing rapidly resulted in a total loss of viability when tested during the first 24 hours after trea ment, partial to total loss in the period from 36 to 60 hours, and positive transfer at 72 hours; (*c*) that the prolongation in the onset of leukemia in tests with rapidly frozen and thawed material resulted from the destruction of cells in numbers insufficient to interfere with the production of leukemia. The leukemic cellular suspensions, when tested before freezing, uniformly resulted, when transplanted to the control recipient mice, in leukemia.

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Oxidative Phosphorylation by Mitochondria of Transplantable Mouse Hepatoma and Mouse Liver

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The synthesis of high energy phosphate bonds as adenosinetriphosphate (ATP) during glycolysis and during certain aerobic oxidations is generally regarded as the probable mechanism mediating much of the energy necessary for cell maintenance and growth. In tumor tissue the phosphorylation mechanism of energy transfer has been identified primarily with the process of glycolysis (6–9) which is well known to be very active in tumors. The extent of oxidative processes in providing phosphate bond energy to tumors, on the other hand, has been less well defined, although there is some direct evidence that oxidative phosphorylations do occur in tumor. Potter (12) observed that the maintenance of the phosphate balance in homogenates of rat hepatoma was somewhat greater during succinate oxidation than in the absence of the substrate. Clowes and Keltch (1) were able to show some net phosphate uptake with homogenates and particles of Walker carcinoma under similar conditions by employing hexokinase and glucose as a trapping agent for the ATP formed. In both studies it was necessary to add fluoride in order to suppress dephosphorylating activities.

One of the principal difficulties in studying oxidations and, thereby, the coupled phosphorylations in tumor homogenates is the presence of large dephosphorylating activities which quickly destroy the ATP added or formed. The apparent deficiency of certain oxidative activities in tumor homogenates (13–16), in contrast to active oxidations obtained with tumor slices (10, 11, 13, 20, 21), has been related by Potter (13) to the excessive breakdown of ATP in the homogenate which in turn leads to disintegration of the enzyme system normally maintained by ATP. Potter and Lyle (16) found that when phosphate breakdown was suppressed by the addition of fluoride, oxalacetate and pyruvate were oxidized by homogenates of Flexner-Jobling carcinoma. The disparity in oxidative activities of tumor homogenates and slices has also been associated with co-factor deficiencies. Weinhouse (20) found that pyruvate, oxalacetate, and a number of other components of the citric acid cycle were oxidized if the homogenate was strongly fortified with diphosphopyridine nucleotide (DPN). This finding is in line with observations that tumor tissues in general are relatively deficient in various co-factors and vitamins (4, 10, 19).

In recent studies in this laboratory on oxidative phosphorylation in liver mitochondria (5), it was observed that maximum rates of net phosphate uptake could be measured *in the absence of fluoride* when extraneous dephosphorylation reactions were minimized by isolating the mitochondria from isotonic sucrose homogenates under conditions which produced little or no adenosinetriphosphatase activity (ATPase) in the mitochondria and little contamination by degradative activities of other cell fractions. By applying these methods of study to a tumor, it has been possible to demonstrate high net rates of ATP synthesis by tumor mitochondria during the oxidation of α-ketoglutarate, glutamate, and succinate. This report presents results of a comparative study of oxidative phosphorylation by mitochondria isolated from transplantable mouse hepatoma and mouse liver.

MATERIALS AND METHODS

Mitochondria were isolated from isotonic sucrose homogenates of mouse liver (C3H strain) and from transplantable mouse hepatoma 98/15 by the method described in a previous paper (5). Mitochondria isolated from 10 gm. of tumor and from 6–7 gm. of liver were suspended in 5 ml. and 10 ml., respectively, of isotonic sucrose. These suspensions were used as enzyme material.

Commercial diphosphopyridine nucleotide (DPN) of 60 per cent purity and a product of 90 per cent purity (3) were used. No differences due to impurities were observed. Other materials and methods employed are reported in the previous work (5).

Phosphorus uptake during oxidation was meas-

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ured as the disappearance of inorganic phosphate from the incubation medium. The phosphorylation system contained the following components unless otherwise specified under individual tables and figures: 0.05 M histidine, 0.04 M KCl, 0.005 M $MgCl_2$, 0.01 M α-ketoglutarate or other substrates in other concentrations, 1.5×10^{-5} M cytochrome c, approximately 0.02 M phosphate, 0.008 M adenosine-5-PO_4 (AMP), 0.025 M glucose, 0.1 ml. hexokinase, 0.3 ml. mitochondria and water in a final volume of 3 ml. The enzyme incubations were carried out with shaking at 28° in air at pH 7.5. In the determination of P:O ratios, O_2 uptake was measured manometrically by the usual Warburg technic, and phosphate was determined in the incubation mixture after removal of protein. In experiments where only rates of phosphorylation were measured, P uptake was followed for 30 to 40 minutes at 10-minute intervals in order to obtain the maximum rate of phosphorylation. Details of the experimental procedure are given elsewhere (5). Maximum rates of P uptake were usually observed in the second 10-minute period. Phosphorylation activities reported in the results are based on these maximum rates.

RESULTS

Characteristics of oxidative phosphorylation in tumor mitochondria.—Freshly prepared mitochondria from transplantable mouse hepatoma esterified large amounts of inorganic phosphate during the oxidation of α-ketoglutarate, glutamate, and succinate in a medium containing the oxidizable substrate, inorganic phosphate, AMP, Mg^{++}, K^+, cytochrome c, hexokinase, and glucose. The time course of phosphorylation during the oxidation of α-ketoglutarate is shown in Chart 1. Maximum rates were noted in the presence of added hexokinase; in the absence of hexokinase, the rate declined as ATP accumulated in the system. These characteristics of phosphorylation are similar to those previously observed with liver mitochondria under the same conditions (5). The phosphate esterified by the tumor particles was shown to be entirely 7′ P[1] transferable to glucose. The utilization of AMP as the external phosphate acceptor, together with the fact that all the 7′ P was transferred to glucose upon addition of hexokinase, indicates that a myokinase enzyme is functioning in tumor mitochondria, as was shown to be the case in liver mitochondria (5). The rate of phosphorylation in the presence of hexokinase was essentially the same for AMP concentrations from 0.002 M to 0.008 M and was also unchanged by substituting adenosinediphosphate (ADP) at a level of 0.002 M for AMP as the external phosphate acceptor.

A significant difference between tumor and liver mitochondria was noted in the greater instability of the phosphorylation system in the tumor, as seen by the greatly reduced rates of phosphorylation during ketoglutarate oxidation in the case of the tumor enzyme which had stood at 0° for several hours (Chart 2). Whereas liver mitochondria suspended in isotonic sucrose retained phosphory-

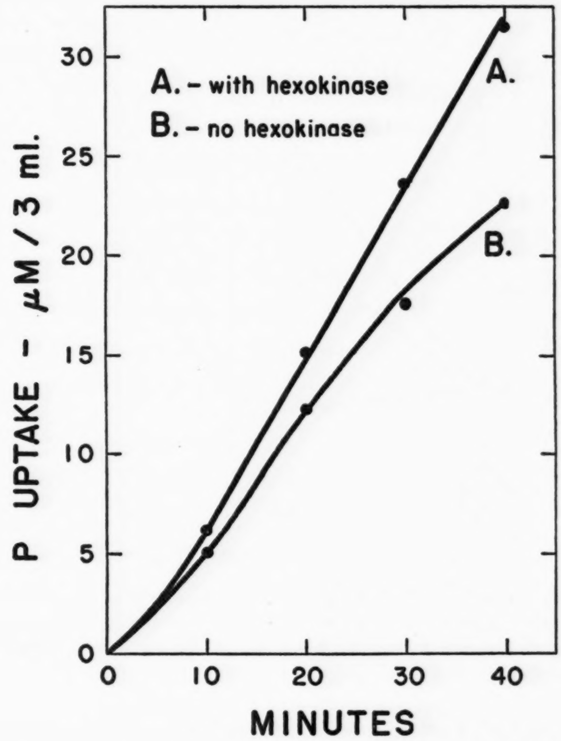

CHART 1.—P uptake during oxidation of α-ketoglutarate by mitochondria isolated from transplantable mouse hepatoma. Other conditions are described in the text.

lating activity for as long as 24 hours at 0° with little loss as seen by the maximum rate attained, tumor mitochondria lost 30–50 per cent of their activity after standing only 2–3 hours at 0°. Tumor mitochondria aged for 24 hours at 0° or for 25 minutes at 28° completely lost their ability to phosphorylate in most cases. The addition of the adenylic nucleotides, AMP, ADP, or ATP, all of which had proved to be effective in preventing extensive loss of phosphorylating activity in the oxidation of α-ketoglutarate by liver mitochondria (5), was without effect in preventing this loss in tumor mitochondria. Subsequent investigation of the rapid loss of activity in the tumor system revealed that one of the essential factors quickly lost from the mitochondria was DPN. Addition of

[1] 7′ P: acid-labile phosphate hydrolyzed to inorganic orthophosphate by 1 N HCl in 7 minutes at 100°.

DPN significantly increased the rate of phosphorylation during the oxidation of α-ketoglutarate and glutamate (Table 1). The effect of DPN on phosphorylation was particularly striking in the oxidation of ketoglutarate by tumor mitochondria, the activity of the aged enzyme being increased four- to fivefold by addition of DPN. Phosphorylation

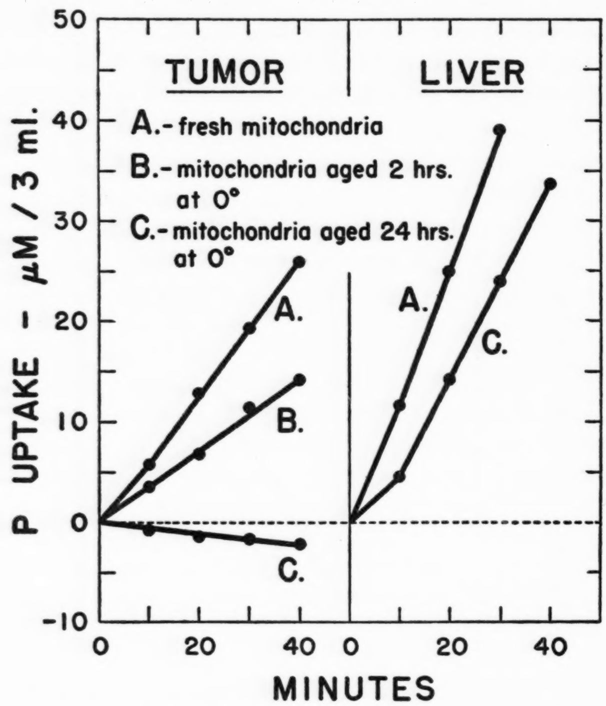

CHART 2.—Effect of aging on P uptake during the oxidation of α-ketoglutarate by mitochondria from transplantable mouse hepatoma and mouse liver. Other conditions are described in the text.

associated with succinate oxidation was not influenced by addition of DPN. Inasmuch as phosphorylation during succinate oxidation was also impaired by short periods of aging at 0° and the original phosphorylation rates associated with ketoglutarate and glutamate oxidation were not completely restored by addition of DPN in most cases, it is apparent that other changes besides loss of DPN have also taken place in the tumor mitochondria. It is seen that DPN caused a greater degree of activation of phosphorylation associated with ketoglutarate oxidation than with glutamate oxidation. This suggests that the affinity of DPN for glutamic dehydrogenase may be somewhat greater than that for ketoglutaric oxidase. The activating effect of DPN was less pronounced in the case of liver mitochondria. Conditions of aging leading to extensive loss of phosphorylating activity in the liver system probably result in far-reaching changes of an irreversible nature, so that the requirement for DPN cannot be as strikingly demonstrated for liver mitochondria as for tumor mitochondria. The marked stimulation of oxidative phosphorylation by the addition of DPN in these experiments with tumor confirms the findings of Weinhouse (20) that the oxidation of many Krebs cycle intermediates in tumor homogenates is favored by the addition of DPN. In the present experiments, it is possible that the DPN level, already low in the tumor, becomes limiting in the mitochondria by diffusion out of the particles during isolation. There is likewise the possibility that a DPN-splitting enzyme may be associated with mitochondria, which reduces the level of the cofactor to limiting concentrations very quickly in the tumor system.

P : O ratios for ketoglutarate and succinate oxidation.—In Table 2, P:O ratios are given for the oxidation of α-ketoglutarate and succinate by freshly prepared mitochondria from tumor and liver. Ratios approaching 3 for ketoglutarate and 2 for succinate were obtained for both tumor and liver mitochondria, indicating that the mechanisms of oxidation and phosphorylation in the above reactions are probably the same in both

TABLE 1

EFFECT OF DPN ON P UPTAKE BY MITOCHONDRIA*

Condition of enzyme	Substrate	0.001 M DPN	Phosphorylating activity (μM P taken up/10 min/mg N) Tumor	Liver
Fresh	0.01 M	−	10.7	16.1
	α-ketoglutarate	+	18.4	18.8
Aged	"	−	3.9	10.2
		+	17.8	11.3
Fresh	0.013 M	−	15.0	23.8
	Glutamate	+	19.7	
Aged	"	−	9.6	15.0
		+	15.7	16.5
Fresh	0.02 M	−	16.4	32.4
	Succinate	+	16.8	
Aged	"	−	12.5	24.8
		+	12.5	

* Substrates (α-ketoglutarate, glutamate and succinate) oxidized by fresh and aged mitochondria isolated from transplantable mouse hepatoma and mouse liver. Tumor mitochondria aged 2 hours at 0°; liver mitochondria aged 3 hours at 5° followed by 24 hours at 0°. Other conditions are described in the text.

tissues. The slightly lower ratios obtained with tumor are probably due to greater instability of the tumor system. Significantly lower ratios were obtained if the tumor particles were allowed to stand for even an hour at 0° before the experiments were performed. The effect of added DPN on the P:O ratio of ketoglutarate oxidation was that of slightly lowering the ratio, although the O_2 and P uptakes were significantly increased.

Ketoglutaric and succinic oxidase activities.—From the data in Table 2 the specific activities of

α-ketoglutaric oxidase and succinoxidase in tumor and liver mitochondria may be calculated and compared. Expressed as μM per hour per milligram of nitrogen, the activities for liver mitochondria are as follows: ketoglutaric oxidase, 19; succinoxidase, 58; for tumor mitochondria these values are 18 and 38, respectively. The specific activities of α-ketoglutaric oxidase in liver and tumor mitochondria were nearly the same, whereas the specific activity of succinoxidase of tumor mitochondria was only 65 per cent of that of liver mitochondria. Similar relative specific activities for the succinoxidase of liver and tumor mitochondria were obtained by Schneider and Hogeboom (17), although their numerical values, obtained at a higher temperature by a method giving maximum activities, were considerably higher. Phosphorylation does not occur, however, during succinate oxidation under conditions for determining maximum activities in the presence of Ca^{++}.

TABLE 2

PHOSPHORUS-OXYGEN RATIOS OBTAINED IN THE OXIDATION OF α-KETOGLUTARATE AND SUCCINATE BY MITOCHONDRIA ISOLATED FROM TRANSPLANTABLE MOUSE HEPATOMA AND MOUSE LIVER*

Mitochondria	Preparation no.	Total N (mg.)	Substrate	Time (min.)	P uptake (μM)	O_2 uptake (μM)	P:O ratio†
Tumor	6–27	0.544	Ketoglutarate	20	13.04	2.94	2.22
"	8–28	0.508	"	15	10.83	2.41	2.25
"	8–28	0.508	" +DPN	15	15.80	3.97	1.99
"	6–27	0.544	Succinate	20	11.13	4.25	1.31
"	8–28	0.508	"	15	15.81	5.12	1.55
Liver	10–10	0.600	Ketoglutarate	15	14.51	3.00	2.42
"	8–21	0.284	Succinate	14	12.20	3.86	1.58

* Substrate concentrations: 0.01 M α-ketoglutarate, 0.02 M succinate. Other conditions are described in the text.

† Each value represents the average of at least two determinations.

Specific phosphorylating activities of tumor and liver mitochondria.—Specific phosphorylating activities of tumor and liver mitochondria during the oxidation of α-ketoglutarate, glutamate, and succinate are given in Table 3. In the oxidation of ketoglutarate, the specific phosphorylating activities of tumor mitochondria fluctuated widely when no DPN was added to the system. Occasionally, high values approaching those of liver mitochondria were obtained, but these were exceptional. The addition of DPN to the tumor system raised the phosphorylating activities to levels approaching those of liver mitochondria. The rate at which mitochondria can form high energy phosphate by oxidation appears to depend upon the level of oxidase present and the P:O ratio of the particular oxidation. Thus, in liver mitochondria the specific phosphorylating activity for succinate oxidation was about 1½ times that for ketoglutarate oxidation, a finding that can be explained by the fact that, although the P:O ratio of succinate oxidation is lower than that of ketoglutarate, the succinoxidase activity was 3 times greater than ketoglutaric oxidase activity in liver mitochondria. In tumor mitochondria, where the succinoxidase was only 65 per cent of that in liver mitochondria, the specific phosphorylating activities with succinate and ketoglutarate were not appreciably different. It should be pointed out, however, that on the basis of the whole tissue the phosphorylating activities for the tumor would be lower than the corresponding activities for liver because of the smaller quantity of mitochondrial substance present in the hepatoma (17).

TABLE 3

SPECIFIC PHOSPHORYLATING ACTIVITIES ASSOCIATED WITH KETOGLUTARATE, GLUTAMATE, AND SUCCINATE OXIDATION OF MITOCHONDRIA FROM TRANSPLANTABLE MOUSE HEPATOMA AND MOUSE LIVER*

	PHOSPHORYLATING ACTIVITY† (μM P TAKEN UP/MIN/MG N)	
SUBSTRATE	Tumor	Liver
0.01 M α-Ketoglutarate	1.65	1.61 (1.88)‡
	0.96	1.76
	1.65	1.84
	1.14	1.71 (2.09)
	0.89	1.65
	1.07 (1.84)‡	1.75
	0.95 (1.56)	1.89
	1.43 (2.08)	
0.013 M Glutamate	1.36	2.38
	1.50 (1.97)	2.42 (2.56)
0.02 M Succinate	2.07	3.24
	1.54	2.69
	1.80	
	1.64	
	1.38	
	2.08	

* Conditions are described in the text.

† The individual activities reported for each substrate represent different enzyme preparations.

‡ The figures in parentheses are corresponding activities determined in the presence of 0.001 M DPN.

DISCUSSION

The presence of active dephosphorylating enzymes in tumor homogenates which prevents

maintenance of ATP necessary for certain Krebs cycle oxidations (13) can be avoided in isolated mitochondria. In the mitochondrial system it has been possible to measure rates and efficiencies of oxidative phosphorylation approaching those of liver mitochondria. Since most of the oxidative activities of tissues studied in relation to intracellular distribution of enzymes have been identified with the mitochondrial fraction (18), it would seem reasonable to assume that the phosphorylating activities coupled with oxidation of mitochondria represent an approximation of the capacity of the tissue to engage in these reactions. On this basis, one cannot consider tumors to be necessarily lagging in these functions. The high rates of oxidative phosphorylation demonstrated in these studies also suggest that respiration plays a greater part in making phosphate bond energy available to tumors than recent evidence would indicate (1, 2).

SUMMARY

It has been shown that an active mechanism for oxidative phosphorylation exists in mitochondria of transplantable mouse hepatoma. Large amounts of inorganic phosphate were taken up with the formation of ATP during the oxidation of α-ketoglutarate, glutamate, and succinate. The phosphorylation system in tumor exhibited more lability than that in liver and showed a more definite requirement for DPN in the case of those substrates the oxidation of which requires this cofactor. P:O ratios were determined for the oxidation of α-ketoglutarate and succinate by tumor and liver mitochondria, but no significant differences due to tissue were observed. The specific activities of ketoglutaric and succinic oxidases were determined under the conditions of active phosphorylation. The specific phosphorylating activities associated with ketoglutarate, glutamate, and succinate oxidations in tumor mitochondria were also determined and compared to those of liver mitochondria. The specific ketoglutaric oxidase activity and the corresponding phosphorylating activity of tumor mitochondria were very nearly equal to those of liver mitochondria, but, in the case of succinate, both activities were less for tumor mitochondria.

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Electrophoretic Changes in Proteins from Livers of Rats Fed 4-Dimethylaminoazobenzene*

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INTRODUCTION

Miller and Miller (3) showed that the carcinogenic dye, 4-dimethylaminoazobenzene, is firmly bound to protein constituents of the rat liver. Such protein-binding reaches a maximum after 4–6 weeks on the azo diet, whereas the neoplasm arises several weeks later. Moreover, the neoplastic tissue contains essentially no protein-bound dye. Hence, Miller and Miller (3) have postulated that certain protein constituents of the cell, which are normally capable of binding the azo dye, are lost or permanently altered in the course of the carcinogenic process. This hypothesis has received support from the recent work of Sorof and Cohen (6), who found that, in consonance with the protein-depletion hypothesis, there is a decrease in the percentage composition of the relatively slow electrophoretic components present in extracts of the neoplastic tissue. In addition, Sorof *et al.* (7) have shown that most of the protein-bound dye migrates with the slow electrophoretic components. Hence, the hypothesis of the loss or alteration of dye-binding protein would seem to be well supported by this as well as other evidence.

The neoplastic type of electrophoretic pattern (increase in percentage composition of faster components with decrease of the slower components) was found by Sorof and Cohen (6) to occur only in the neoplastic tissue itself, and not in the adjacent normal liver tissue. In the present experiments we have confirmed the alteration of electrophoretic components as described by Sorof and Cohen. However, with the preparative method here used, which differs in several respects from that of Sorof and Cohen, the livers of rats fed the noncarcinogenic azo dye, 4-aminoazobenzene, and also the livers of young rapidly growing rats, show the same type of electrophoretic pattern found in the neoplastic tissue. In our animals, too, the preneoplastic livers show the same electrophoretic changes as the later neoplastic stages. Under the conditions of the present experiments, the decrease in percentage composition of the slow components occurs independently of the carcinogen and of the neoplastic state. The present results suggest that the type of electrophoretic alteration described is possibly characteristic of rapidly growing tissues or of tissues in the process of vigorous protein synthesis.

METHODS

Young female Long-Evans rats with an average weight of 212 gm. each were maintained on an experimental diet consisting of a mixture of 50 per cent potato flour, 49.94 per cent stock rat diet,[1] and 0.06 per cent (weight) 4-dimethylaminoazobenzene (DAB). The dye, dissolved in 100 ml. of warmed olive oil, was added to 5 kg. of total mixture. The basal or normal diet was the same as the above minus the DAB. Two groups of ten animals each were placed on the experimental and the basal diet for periods of 5, 10, 14, and 16 weeks; various control groups other than those on the basal diet were as described below. The animals were sacrificed after light ether anesthesia by cutting the neck veins and arteries. Livers were perfused *in situ* through the hepatic portal vein with ice-cold saline, with the use of a Valentine irrigator adjusted to a hydrostatic pressure of 30 inches of water, until the liver lobes showed no traces of blood. After being rinsed in water, the livers were stored at −18° C. The animals were killed without fasting in order to retain as much liver protein as possible (2).

In addition to the above animals maintained on the basal diet, the following five control groups were studied: (*a*) Rats were fed the basal diet plus 0.06 per cent 4-aminoazobenzene AB (AB, noncarcinogenic) and sacrificed after 5 weeks. (*b*) A second group was fed the DAB diet for 10 weeks,

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[1] Stock rat diet: 50 parts corn meal, 50 parts wheat flour, 25 parts linseed meal, 6 parts alfalfa meal, 6 parts casein, 6 parts milk powder, 5 parts scratch feed, 4 parts chalk. All parts by weight.

and then put on basal diet for 3 weeks. (*c*) A third group was maintained on the DAB diet for 14 weeks, followed by 2 weeks on the basal diet. (*d*) A fourth group received the DAB diet for 16 weeks, followed by 2 weeks on the basal diet. (*e*) Finally, the livers of rapidly growing young animals (8 and 18 days *post partum* rats) were compared to the livers of the above groups. All animals were caged in groups of four and were permitted to take food and water ad libitum. There was no significant weight change at 5 weeks. By the tenth week of DAB diet, the animals had lost approximately 5 per cent of their body weight. No further decrease occurred at 14–16 weeks. Animals on basal diet showed an average weight gain of 5 per cent.

Preparation of extracts and electrophoresis.—The livers of all groups were treated in the following manner: Samples of liver tissue, each weighing 200–400 mg., were removed from the ten livers comprising an experimental group. Two gm. were placed in a chilled Potter-Elvehjem tissue homogenizer with 5 ml. 0.024 M veronal buffer, pH 8.1; the low concentration of alkaline veronal buffer permits maximal protein extraction (5). After thorough homogenization, the highly viscous preparation was dialyzed for 15–20 hours against 0.144 M buffer at pH 8.45 (0.12 M NaCl, 0.024 M veronal), then centrifuged twice at 15,000 *g* for 30 minutes to remove all insoluble material. The supernatant fluid was then diluted 1:1 with buffer.[2] After preliminary trials, electrophoresis was standardized at 60 minutes (9 v/centimeter in a 2-ml. cell; specific conductivity of buffer, 7.03×10^{-3} mho at 1° C.). The electrophoretic patterns were enlarged about 5 times, and the relative percentage composition was determined by the method suggested by Tiselius and Kabat (8). Mobilities were calculated from the epsilon peak. The descending patterns were used for all measurements.

RESULTS

Histological sections of livers from animals on the DAB diets were compared with sections on the basal diets.[3] No differences were noted in the livers of animals maintained for 5 weeks on the DAB diet. At 10 weeks, however, proliferating areas of small, densely staining bile duct cells were evident. In the 14–16-week livers, replacement of hepatic tissue by bile duct proliferations was much more pronounced, especially around the portal veins, as described by Orr (4). On the whole, the gross appearance of the liver was a good indication of the extent of proliferative tissue. Specimens with a few small nodular areas showed relatively little cholangiomatous proliferation in sections, whereas greater numbers of gross nodules were associated with more extensive cholangioma. At the 10-week stage, livers from DAB-fed animals may show cholangioma in regions of the liver apparently free from visible nodules. True hepatomas, as distinct from cholangiomas, were relatively infrequent, as were cirrhotic livers.

Table 1 summarizes the results with respect to areas of the components, and Charts 1 and 2 show the essential alterations of the patterns of controls and experimentals. Each average represents two or more extractions, and each extraction was subjected to two electrophoretic runs. The basal diet livers gave consistently reproducible composition values after 5, 10, and 14 weeks on the diet. Component 1 actually consists of three or more electrophoretic species, but we consider it preferable to include them all under component 1 for the sake of objective treatment of the data, rather than to subdivide it into components which almost entirely overlap one another. The mobility of the dominant electrophoretic species of component 1 is 5.27×10^{-5} cm²/v/sec. A small component, comprising 1–3 per cent and moving with a mobility of about 11.0×10^{-5} cm²/v/sec, occurred in front of component 1. This peak was not included in the area measurements because of its small size and considerable spread. There were no apparent significant changes related to this fast component; however, it would be difficult to detect changes in so small a component.

Component 1 seems to increase in livers containing large proportions of neoplastic tissue, and in 18-day rat liver. The changes, as may be seen from Table 1, are not, however, altogether consistent. The 14–16-week DAB livers show increases in this component which may or may not revert to its normal percentage when the animals are placed on the normal (basal) diet.

Components 2 and 3 are quite clearly defined, and changes in proportions of these components in livers of the DAB-fed and basal animals are statistically significant; in fact, one can readily separate the DAB from the basal patterns by inspection (Chart 1). The dominant electrophoretic species of components 2 and 3 migrate at 3.60 and 2.07×10^{-5} cm²/v/sec, respectively. As will be seen in Chart 1, considerable protein remains in the starting boundary (epsilon). Component 2 migrates as a relatively homogeneous area and main-

[2] The concentration of protein in the supernatant fluid was determined with a Bausch and Lomb "Abbe" Refractometer and was found to be 1.5–2.3 per cent.

[3] We are indebted to Professor R. Kinosita for advice on various matters pertaining to this work and for examination of some of our histological sections.

tains its definite and symmetrical shape even after prolonged electrophoresis. Nevertheless, it must be kept in mind that even such a relatively uniform component as 2 may represent several different proteins having similar mobilities, as shown by the work of Gjessing, Floyd, and Chanutin (1).

In general, after 5 weeks or more of DAB feeding component 2 increases significantly, and component 3 decreases when compared to the animals on the basal diet (Chart 3). In the 10-week DAB-fed animals, the electrophoretic patterns fall into tions of the several components (Table 1, Chart 1). The altered percentage of electrophoretic components observed in extracts of neoplastic livers is therefore not exclusively characteristic of the neoplastic state.

In three groups of rats, feeding of DAB was discontinued after 10, 14, and 16 weeks, and the animals were maintained on the basal diet for periods of 2 or 3 weeks. The modified electrophoretic patterns did not revert to the normal with the elimination of the DAB diet. A short period of 2 weeks on

TABLE 1

RELATIVE ELECTROPHORETIC COMPOSITION OF EXTRACTS FROM LIVERS OF RATS FED 4-DIMETHYLAMINOAZOBENZENE (DAB) AND OF CONTROLS (BASAL DIET)

PERIOD	DIET	NO. OF DETER.	NO. OF RATS	COMPONENTS (PER CENT) No. 1	No. 2	No. 3
				5.27± 0.34*	3.60± 0.32*	2.07± 0.15*
5 weeks	Basal	6	24	17.88± 1.65	27.51± 2.03	54.60± 2.66
5 weeks	DAB	7	20	17.08± 3.04	39.68± 5.23	42.48± 4.57
10 weeks	Basal	4	12	16.25± 2.03	26.97± 2.28	56.86± 0.95
10 weeks†	DAB *A*	4	24	23.30± 0.82	37.45± 6.15	39.25± 6.12
	B	6	24	12.96± 0.29	63.93± 2.96	23.10± 3.03
14 weeks	Basal	2	12	17.33± 0.61	30.70± 1.85	51.97± 2.01
14 weeks†	DAB *A*	4	24	16.81± 2.72	40.76± 5.92	42.42± 3.45
	B	4	24	14.05± 1.05	63.19± 2.25	22.76± 1.86
14 weeks‡	DAB	6	24	21.04± 4.45	40.95±12.20	37.97±10.40
16 weeks‡	DAB	2	4	19.35	40.25	40.40
16 weeks‡	DAB	4	5	23.20± 0.29	45.19± 5.90	31.61± 7.01
5 weeks	AB	4	5	18.85± 3.18	36.47± 1.72	45.42± 3.63
10 weeks	DAB & 3 weeks Basal	4	10	14.85± 3.26	38.32± 3.92	46.82± 6.35
14 weeks†	DAB & 2 weeks Basal	4	10	16.76± 0.62	35.72± 2.29	41.51± 2.62
16 weeks†	DAB & 2 weeks Basal	4	5	15.70± 2.74	44.02± 4.13	40.25± 2.11
16 weeks‡	DAB & 2 weeks Basal	4	5	20.15± 2.42	38.00± 4.28	41.85± 2.88
8 days	*Post partum*	2	18	16.05	38.97	44.97
18 days	*Post partum*	4	7	23.46± 1.10	34.73± 6.13	41.80± 6.44

* Average mobilities and standard deviations. Standard deviation $= \pm\sqrt{\frac{(d^2)}{n-1}}$

† No tumor nodules visible *macroscopically*.

‡ Livers contained numerous microscopic nodules.

two groups (labeled *A* and *B* in Table 1). Type *B* livers show much more extensive alterations in the percentages of components 2 and 3 (Table 1). Types *A* and *B* are apparently not extremes of a range as is shown by their standard deviations. A similar dichotomy exists in the data for the 14-week DAB livers. Thus far, in advanced stages of neoplastic development, when numerous large nodules are macroscopically visible, the extensive type of alteration (Type *B*) has not been found, all specimens showing the moderate alteration (Type *A*). The increase in component 2 and decrease in component 3 after 5 weeks of DAB diet are also seen in livers not affected by carcinogens. Modifications of similar magnitude occur in the rats fed the noncarcinogenic 4-aminoazobenzene (AB). Also, electrophoretic patterns from the livers of young (8- and 18-day) rats showed similar propor- the basal diet might be expected to induce only a partial reversion to normal; however, considering the range of variability, there is no evidence that reversion to the basal diet produced any tendency toward the normal electrophoretic pattern.

DISCUSSION

The present studies, although carried out in complete independence, are in close agreement with those of Sorof and Cohen (6), who found that extracts of DAB-induced hepatoma show a significant increase in the faster moving components and a decrease in the slower ones, as compared to normal livers. This type of neoplastic pattern did not occur in Sorof and Cohen's "preneoplastic" liver nor in normal tissue adjacent to the neoplasm. In the present experiments, the neoplastic electrophoretic change was constantly observed in

livers from all DAB animals, including those which received the dye for only 5 weeks. We are not prepared to refer to these early stages as "preneoplastic," since histological sections frequently reveal proliferating foci in macroscopically normal regions of the liver as early as 10 weeks. However,

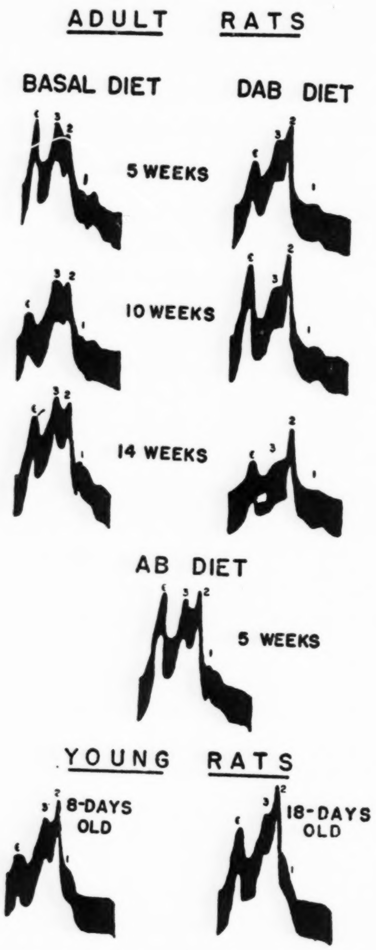

CHART 1.—Typical electrophoretic patterns of various liver extracts. DAB diet refers to basal diet plus 0.06 per cent 4-dimethylaminoazobenzene. AAB diet is basal diet plus 0.06 per cent 4-aminoazobenzene. All patterns made after 60 minutes in 2-ml. Perkin-Elmer cell, μ = 0.144 M, pH 8.4, 9 v/cm. Numbers refer to components in Table 1. Note that DAB patterns can be distinguished from normal (basal) patterns by direct inspection.

the characteristic neoplastic pattern occurs in 5-week DAB livers in which neoplastic tissue is ostensibly absent or at least very difficult to detect. We must therefore conclude that the so-called neoplastic pattern is also associated with the preneoplastic condition.

This apparent discrepancy between our data and those of Sorof and Cohen may be attributable to any one or all of several variables. The livers used by us were frozen, and the total homogenate was extracted; on the other hand, Sorof and Cohen used fresh liver and centrifuged the homogenate so that the particulate matter contributed less soluble constituents than in the present preparation. In addition, the number of animals used in the present experiments was greater, the diet was different, and female rats were used rather than males, as in the experiments of Sorof and Cohen. It remains for future experiments to determine whether such variables may account for the discrepant results.

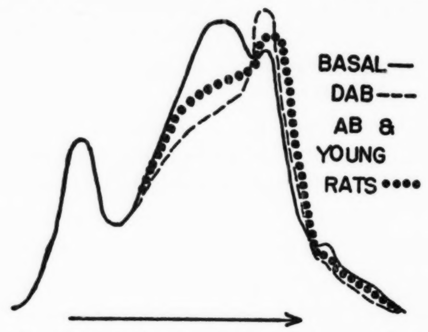

CHART 2.—Schematic comparison between electrophoretic patterns of various rat liver extracts. Prepared from descending patterns only. Patterns from animals fed noncarcinogenic dye and from young animals (dotted line) are similar although electrophoretic patterns are possibly less pronounced than in the DAB fed animals (------).

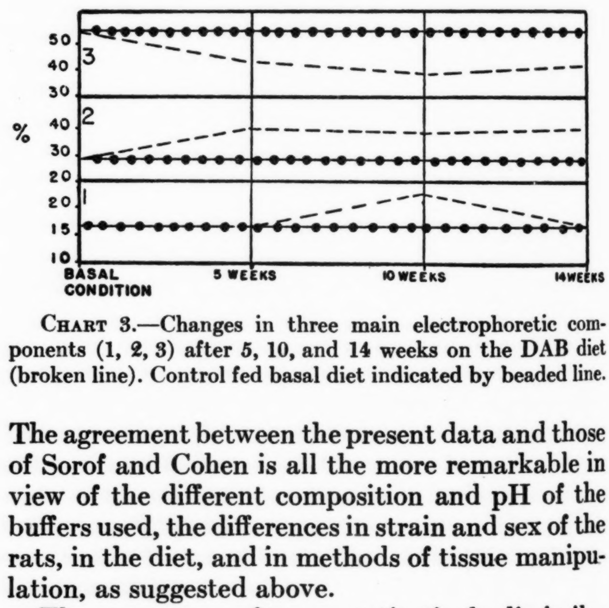

CHART 3.—Changes in three main electrophoretic components (1, 2, 3) after 5, 10, and 14 weeks on the DAB diet (broken line). Control fed basal diet indicated by beaded line.

The agreement between the present data and those of Sorof and Cohen is all the more remarkable in view of the different composition and pH of the buffers used, the differences in strain and sex of the rats, in the diet, and in methods of tissue manipulation, as suggested above.

The appearance of two quantitatively dissimilar electrophoretic patterns in animals fed DAB for 10 weeks or for 14 weeks (Table 1, types *A* and *B*) has not to our knowledge been described. Type *A* livers show moderate alterations of the three main groups of electrophoretic components, whereas those of type *B* show much more pronounced alterations. The standard deviations of the two groups suggest that they are not merely extremes of the variation range. These two types of patterns were observed in livers which showed no macroscopic tu-

mor nodules—in other words, in livers which were either in early stages of neoplastic growth or in the preneoplastic period. On the other hand, livers containing numerous macroscopic nodules gave much more uniform electrophoretic patterns. This suggests that early events of the neoplastic process include an exaggerated (type *B*) electrophoretic response. That this exaggerated response probably occurs in the preneoplastic period is suggested by the fact that livers with numerous macroscopic nodules usually show the moderate (type *A*) response.

Miller and Miller (3) showed that DAB is firmly bound to liver proteins and that such binding is maximal after 4–6 weeks on the DAB diet. However, neoplastic tissue arising in such livers shows no perceptible bound dye; hence, the authors hypothesized that the carcinogenic process involves the elimination of protein constituents capable of binding the dye. Sorof *et al.* (7) provided supporting evidence for the hypothesis of protein loss by showing that slower electrophoretic components are reduced in neoplastic tissue and that these slower components apparently bind a major portion of the protein-bound dye. The present results throw some doubt on the possible causal relationship between dye binding by the liver proteins on the one hand and reduction of slow electrophoretic components on the other. As shown in Table 1, the slower components are also reduced in animals fed the noncarcinogenic aminoazobenzene for only 5 weeks. Moreover, the rapidly growing normal livers of 8- and 18-day-old rats (Table 1) show similar electrophoretic changes. This suggests that the observed type of electrophoretic pattern is probably associated with a more inclusive phenomenon than carcinogenesis. From the observations that it occurs in the rapidly growing organs and in animals fed aminoazobenzene, as well as in neoplastic tissue, we are led to the hypothesis that a shift in percentage composition such that faster electrophoretic components are increased, is associated with rapid protein synthesis, common to rapid embryonic growth, regenerating tissue, and functional phases of certain secretory and nervous tissues.

SUMMARY

1. Electrophoretic patterns of liver extracts from adult female rats fed a standard ration containing 4-dimethylaminoazobenzene show distinct and consistent differences from control animals maintained on the same diet minus the azo dye. Animals fed the azo diet for 5, 10, and 14 or 16 weeks show a significant reduction in percentage of the slower electrophoretic components and an increase in the faster components.

2. Similar electrophoretic alterations occur in liver extracts of animals fed 4-aminoazobenzene (noncarcinogenic), and similar patterns were obtained from the livers of young, rapidly growing animals.

3. The hypothesis is advanced that the observed type of electrophoretic pattern is common to rapidly growing tissues and tissues carrying on vigorous protein synthesis.

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Inhibition of Human Mammary and Prostatic Cancers by Adrenalectomy*

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It is known that excision of the adrenal glands with maintenance of the animal on sodium chloride has two opposite effects on tumors. Adrenalectomy stimulates the growth of lymphoid tumors in the rat and mouse while retarding the growth rate of many other neoplasms in these species; a decrease in the size of established tumors has not been reported. It will be demonstrated in this paper that adrenalectomy with maintenance of the organism on cortisone acetate can cause some regression of neoplasms, namely, certain cancers of the breast and prostate of man.

The depression of the growth rate of transplantable tumors by adrenalectomy has been observed several times. Joannovics (23) observed that transplanted sarcomas in adrenalectomized mice were slightly over 20 per cent less in weight than in intact controls. Roffo (30) reported a decrease in the growth rate of a transplanted sarcoma and carcinoma of rats following excision of the adrenals. Bischoff and Maxwell (2) did not find that adrenalectomy significantly affected the growth behavior of Walker carcinoma 256 in rats. However, Ingle and Baker (21), with more precise methods, found that adrenalectomy significantly retarded the rate of tumor growth of Walker carcinoma 256 in force-fed ovariectomized rats and in intact males; in this laboratory we have been able to confirm these findings. Funk *et al.* (10) also found that the Walker tumor grows at a reduced rate in adrenalectomized rats. Ingle and Baker ascribed the retardation of neoplastic growth to the theory that rapidly growing tissues cannot attain a "peak rate of anabolism" in adrenally insufficient animals.

Apart from rodents, the only observations on the growth of tumors in adrenalectomized creatures have been on a human neoplasm. Huggins and Scott (20) demonstrated retardation of growth in a man with prostatic cancer after total adrenalectomy, and Cox (4) later observed "a dramatic if temporary clinical improvement" in a patient with this tumor after subtotal removal of the adrenals.

Acceleration of the growth of neoplasms by adrenalectomy was discovered by Murphy and Sturm (27, 32), who found that the number of "takes" of a transplantable lymphatic leukemia was greatly increased and the length of life of successfully inoculated rats was less following adrenalectomy than in intact rats. Law *et al.* (25) observed that adrenalectomy considerably increased the incidence and the time of appearance of spontaneous lymphoid leukemia in C58 mice.

Heilman and Kendall (12) found that the administration of 11-dehydro-17-hydroxycorticosterone caused a rapid, profound, yet temporary regression of a transplanted lymphosarcoma in mice; furthermore, when injections of this compound were begun soon after inoculation of the tumor, its growth was delayed for as long as Compound E was given. The growth rate of the following transplantable tumors has been depressed after the injection of 11-oxygenated steroids: three types of lymphosarcoma (33, 7); two osteogenic sarcomas (33); rhabdomyosarcoma (13); ependymoma (3); and various other sarcomas (33). Ingle, Prestrud, and Baker (22) found that cortisone acetate caused a suppression of the growth rate of the Walker 256 tumor in tube-fed rats.

Pituitary adrenocorticotrophin (ACTH) and cortisone acetate have been administered to patients with malignant disease. Pearson *et al.* (28) observed a dramatic and progressive but temporary decrease in the size of enlarged lymph nodes

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and the spleen of six patients with lymphomatous tumors after the administration of these agents; there was no obvious clinical response in two patients with metastatic cancer of the breast and prostate, respectively. Eliel *et al.* (6) reported no demonstrable shrinkage of tumors in seven patients with advanced carcinoma of various kinds following treatment with ACTH or cortisone acetate. Postlethwait *et al.* (29) found that the course of malignant disease appeared to be completely unaltered by cortisone in nine patients with advanced carcinoma of the digestive tract. Spies *et al.* (31) stated that there was a reduction in size and a decrease in pain in a patient with carcinoma of the lip following ACTH; no histologic modifications were detected in the tumor in this case. Taylor *et al.* (34) treated 26 patients who had advanced neoplastic diseases with cortisone acetate or ACTH and were able to confirm the finding of regressions of lymphomatous tumors observed earlier; most of the patients manifested striking temporary improvement in their general condition with lessening of fever, when present, increased appetite, and improved strength and sense of well-being, but no control of the carcinoma was observed.

METHODS

Bilateral adrenalectomy was carried out on eighteen patients with the following neoplasms: prostate, seven cases; breast, seven; miscellaneous cancers, four. All the patients had far advanced neoplasms with extensive metastases.

The gonads of all the patients with mammary or prostatic cancers had been excised prior to adrenalectomy except in one woman who was in the postmenopausal state. All the prostatic cases had had a clinical remission induced by orchiectomy with a subsequent relapse; all had been treated in addition with estrogenic substances, mostly diethylstilbestrol, for long periods. These patients have been observed for 4–9 months after adrenalectomy. The miscellaneous cancers were squamous carcinoma of the urethra, melanosarcoma, chorionic epithelioma, and an undifferentiated carcinoma, presumably of pulmonary origin. These patients were studied over periods of 1–3 months after adrenal excision.

We have published (17) the medical and surgical methods involved in one-stage bilateral adrenalectomy in man and maintenance of these patients by medicines. These technics were followed precisely in this series. No substances with hormonal activity were administered except cortisone acetate and desoxycorticosterone acetate (DOCA).

The total protein content of the least thermocoagulable percentage of serum (16, 18) was determined twice each week. Acid and alkaline phosphatases were measured by the method of King and Armstrong (24); glucose tolerance and insulin tolerance (9) and the water diuresis test (26) were determined pre- and post-operatively in all cases.

RESULTS

Metabolic and clinical status of the adrenal-less man.—There were two post-operative deaths in this series of eighteen adrenalectomized patients with cancer. However, bilateral adrenalectomy has now been carried out by us for one or another reason in 29 consecutive cases without operative fatality.

Following the rather large replacement doses of cortisone acetate and DOCA required to insure the prevention of adrenal insufficiency during and after adrenalectomy, the patient within a week was placed on a hormonal substitution program which in most cases proved to be the future maintenance regimen. Criteria for adequate substitution were the prevention of any sign or symptom of adrenal insufficiency. The maintenance of electrolyte and carbohydrate balance and of adequate blood pressure without orthostatic hypotension were the best indicators.

Cortisone has made possible this satisfactory replacement regimen. In our experience 25 mg. of cortisone acetate by mouth twice daily and the oral ingestion of 3 gm. of sodium chloride in most cases gave excellent results. Some of our patients have been maintained on as low as 12.5 mg. cortisone acetate twice a day with 3 gm. of salt, while other patients have required 2–4 mg. of DOCA daily in addition to cortisone acetate and salt to prevent orthostatic hypotension. The orthostatic hypotension occasionally seen was not corrected by augmenting the sodium chloride intake or even raising cortisone acetate to above 75 mg. a day. To increase the cortisone acetate above 50 mg. a day does not seem wise, for at these levels the protein catabolic effect becomes too predominant. The majority of the cancer patients after operation have an excellent appetite and gain weight rapidly.

When the patient is on an adequate hormonal maintenance, the glucose tolerance test, insulin tolerance test, and water diuresis test are normal and similar to those in the pre-operative period. In two patients with diabetes mellitus there has been no significant change in the insulin requirement.

When maintained adequately, adrenalectomized patients have a healthy appearance, are not incapacitated, and they are able to engage in all their usual activities. Cutaneous pigmentation of the addisonian type does not occur. Six of the pa-

tients, including four males and two females, had hot flushes, but these vasomotor changes disappeared within 3 months. The patients withstand well the common ordeals of life. Under severe emergencies, such as extreme heat and severe infections, signs of adrenal insufficiency develop occasionally, but these were easily eliminated by increasing the amount of cortisone acetate and DOCA.

On our hormonal maintenance schedule the total protein content and least thermocoagulable protein percentage of serum can reach normal values and the erythrocyte number and hemoglobin content of whole blood as well.

Adrenalectomy for cancer of the prostate.—In this series of seven patients there was one post-operative death (Case 3), leaving six effective cases of prostatic cancer. Another patient (Case 4) died of acute pulmonary edema 49 days after adrenalectomy.

In four of the patients the primary tumor was large and nodular; the neoplasm decreased appreciably, as determined by palpation in two of these men, while in two other cases it was unaffected. In two cases in this series the prostate was atrophic at the time of adrenalectomy because of previous anti-androgenic control, and the gland underwent no further change; both of these patients were incapacitated because of metastatic lesions.

All the patients gained weight after adrenalectomy. In five patients the hemoglobin concentration (Table 1) increased, and in three patients there was an increase in the total number of erythrocytes. In three patients there was an increase in the total protein concentration (Chart 1), and in four patients there was a decrease of the least coagulable percentage of serum.

A large increase of acid phosphatase in serum was present before adrenalectomy only in two patients; in both cases there was a significant decrease in the level of this enzyme (Chart 2), but the decrease was not so precipitous as usually occurs in a previously untreated case of prostatic cancer after orchiectomy.

Perhaps the most striking observation has been the immediate and persistent relief of crippling pain in the bones. All the six patients in this series suffered from this complaint, and the pain was of such severity that all of them had been treated by morphine or codeine prior to adrenalectomy. In one patient (Case 7) there was no diminution of pain; in five patients the pain was completely relieved within 2 days after adrenalectomy, and the relief has persisted during the observational period. We are of the opinion that three of the six patients (Cases 2, 5, 6) in this series have had a clinical remission of the disease of considerable magnitude.

Adrenalectomy for mammary cancer.—This procedure was carried out on seven patients in this

TABLE 1

WHOLE BLOOD AND BLOOD SERUM VALUES AFTER ADRENALECTOMY IN CANCER

Case		Days	Hemoglobin gm/100 ml	Erythrocytes $\times 10^6$/c. mm	Total protein gm/100 ml	Least coagulable (per cent)	Serum phosphatases acid units/100 ml	Serum phosphatases alkaline units/100 ml
PROSTATIC CANCER								
1. E.S.	Pre-operative		13	4.37	6.90	23	4.0	11.1
	Post-operative	251	12	3.82	7.47	20	6.9	11.6
2. C.F.	Pre-operative		13.4	4.19	6.40	26	18.9	26.2
	Post-operative	240	17.4	5.23	7.38	20	7.0	11.1
3. J.S.	Pre-operative		10.5	3.89	7.30	24	4.4	12.3
4. L.S.	Pre-operative		12.2	4.24	7.09	26	4.5	8.5
	Post-operative	47	13.5	4.12	6.31	25	3.4	4.6
5. W.M.	Pre-operative		11	3.16	6.13	30	92.8	30.6
	Post-operative	180	13	4.03	7.11	20	32.3	33.9
6. J.F.	Pre-operative		12.2	4.02	6.28	24	4.0	6.8
	Post-operative	158	13.3	4.13	7.13	18	3.0	7.0
7. G.H.	Pre-operative		8.0	3.26	6.92	32	4.3	22.5
	Post-operative	137	9.5	2.67	5.59	41	2.0	12.8
MAMMARY CANCER								
8. L.C.	Pre-operative		12.2	3.91	5.23	27	6.7	6.0
9. M.B.	Pre-operative		13	3.99	7.08	23	4.2	10.7
	Post-operative	168	13.5	4.13	5.99	24	6.4	12.6
10. R.B.	Pre-operative		11.5	4.0	6.22	28	0.8	7.2
	Post-operative	19	13.0	4.4	6.12	38	2.6	6.3
11. J.B.	Pre-operative		10.6	3.47	5.77	29	4.4	5.6
	Post-operative	156	12.0	4.02	5.91	30	1.7	9.6
12. G.N.	Pre-operative		12.9	4.41	6.21	26	4.6	9.9
	Post-operative	155	11.5	3.80	6.38	22	6.0	8.1
13. N.C.	Pre-operative		15	5.04	6.00	22	3.4	4.6
	Post-operative	117	12.4	4.01	6.65	20	4.2	6.0

category. There was one post-operative death (Case 8) leaving six effective cases. One patient (Case 10) died 23 days after adrenalectomy.

Among the six effective cases, the course of the disease was not influenced favorably in two patients (Cases 10, 11); in one case there was minor improvement (Case 9); some regression of the lesions was observed in three cases (Cases 12, 13, 14).

In Case 10 there were extensive pulmonary metastases, and the patient suffered from dyspnea; the lesions seemed to be completely unaffected by adrenalectomy. In Case 11, there were large sloughing ulcerations of cancers of each breast; following adrenalectomy the ulcerative process advanced rapidly.

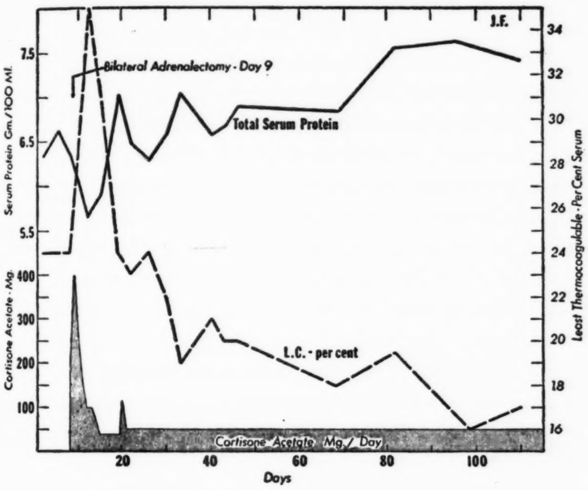

CHART 1.—Increase of total protein and decrease of least percentage of thermocoagulable protein of serum in a patient (Case 6) with metastatic cancer of the prostate. L.C. = Least thermocoagulable protein of serum.

One patient (Case 9) was moderately improved in that pain decreased and body weight increased after adrenalectomy and excision of the ovaries. The osseous metastases in this person had been advancing rapidly prior to adrenalectomy. Following removal of gonads and adrenals the advance seems to have been retarded, and there has been slight and partial recalcification of the lesions.

In two patients (Cases 12, 13), both with pulmonary metastases, there has been an increase in the total serum proteins together with a decrease in least coagulable percentage of serum and regression of pulmonary metastases as determined by radiography. In a man (Case 13) with evidence of intracranial metastases and large pulmonary metastases as well, there was a significant decrease (Figs. 1, 2) of both lesions within 3 weeks after adrenalectomy, and the gains are still maintained after 4 months.

In one woman (Case 14) there was a decrease (Figs. 3, 4) in the size of cutaneous metastases of mammary cancer in a post-mastectomy scar noted within 2 weeks after adrenalectomy and in a large tumor of the contralateral breast also. The regression was accompanied by evidence of involution as determined by cytologic methods on biopsy specimens (Figs. 5, 6).

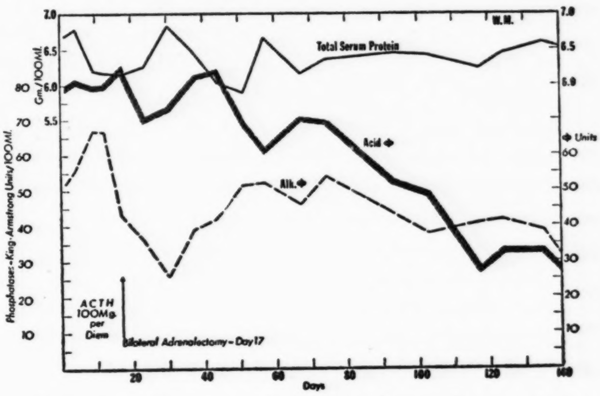

CHART 2.—Decrease of level of acid phosphatase of serum after adrenalectomy in a patient (Case 5) with metastatic cancer of the prostate.

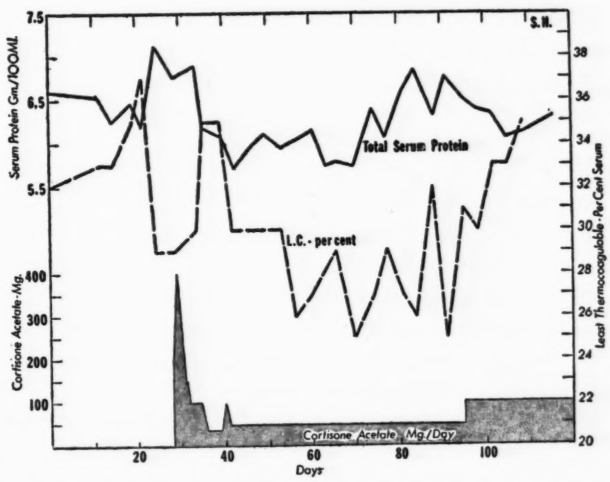

CHART 3.—Decrease of total protein and increase of least percentage of thermocoagulable protein of serum at cortisone acetate levels above 50 mg. daily in a patient with prostatic cancer.

Adrenalectomy for miscellaneous tumors.—There were four patients in this series with advanced cancer (squamous carcinoma, melanosarcoma, chorionic epithelioma, and an undifferentiated carcinoma) other than that of the breast and prostate. All had extensive metastases. They have been followed for 1–3 months after adrenalectomy. Adrenalectomy had no detectable effect in retarding the growth of these tumors. To the contrary, positive evidence was obtained of increase in size of the cancers in all of them.

Treatment of prostatic cancer by cortisone acetate and ACTH.—Pituitary adrenocorticotrophin (ACTH), 100 mg. daily for 14 days, was administered to three patients with advanced cancer of the prostate with metastases. Cortisone acetate was injected intramuscularly in four similar cases for periods up to 90 days, the dosage being exactly that used in the medical management of the patients subjected to bilateral adrenalectomy.

There was a considerable uniformity of response to ACTH and cortisone acetate. The findings of Taylor *et al.* (34) were confirmed in that the patients had an improved sense of well-being, an increase of appetite, but no decrease in the size or activity of the neoplasms. Bone pain was lessened by an estimated 50 per cent, but in no case was pain eliminated for more than 1 or 2 days. At a level of cortisone acetate, 100 mg. daily, or higher, there was in each person a decrease in total protein content and an increase in the least coagulable percentage of serum (Fig. 7).

Two of the patients who obtained partial relief of pain from administration of cortisone acetate were treated subsequently by adrenalectomy with complete relief of bone pain at maintenance levels of cortisone acetate of 37.5–50 mg. daily. In both of these patients there was detectable regression of the neoplasm after adrenalectomy.

DISCUSSION

In the earlier observations on the effect of adrenalectomy on neoplastic growth, a retardation of the rate of growth of some tumors had been reported. It is impossible to prove in any given single case that the growth of a human cancer has been merely slowed. However, regression is easy to demonstrate, and this occurs in some human neoplasms after adrenalectomy.

It is of interest that bilateral adrenalectomy can induce regression of certain malignant tumors and yet is unable to influence favorably other neoplasms having the same site of origin. Evidently the functional characteristics of neoplasms vary greatly.

It should be pointed out that in this small series of patients only prostatic and mammary carcinomas, and not all of them, underwent some regression after adrenalectomy. The "miscellaneous tumors"—squamous carcinoma, melanosarcoma, chorionic epithelioma, and an undifferentiated carcinoma—were not affected. Now it is established that sex hormones of various kinds increase the activity or cause regression of certain far advanced tumors of the prostate (19) and the male (8) and female mammary glands (1, 11). The cases in which adrenalectomy induced regression comprise tumors which already are known to be influenced in an important manner by sex hormones.

The mechanism whereby regression of some tumors occurs after adrenalectomy is not clearly established, largely because of uncertainty about normal adrenal function. For instance, there is no general agreement concerning what hormones the normal adrenal produces, in what quantity, or whether their number is few or many. We have shown (17) that the syndromes resulting from hyperfunctioning adrenal cortical tumors can be explained on the basis of androgenic, estrogenic, or corticoid effects, sometimes alone and other times in varying combinations and proportions.

Since regressions occur after adrenalectomy with maintenance of the patient on cortisone acetate, it may be inferred that steroids of this type are not responsible for continuing functional activity of the neoplasm. It is most reasonable to assume that the regressions are due at least in part to elimination of critical amounts of sex hormones. It must be pointed out, however, that adrenalectomy seems to have a nonspecific effect in retarding growth of several transplantable tumors of rodents, and this phenomenon may be partially operative in prostatic and mammary cancers.

It is known that the anti-androgenic treatment of prostatic cancer through estrogen administration or orchiectomy has three effects on prostatic cancer: (*a*) No retardation of the growth rate occurs in a small percentage of patients; (*b*) Extensive regression of primary and metastatic lesions is observed in something more than one-half of the cases; (*c*) Regression of the primary tumor with progression of the metastasis (14, 15). In category *c*, clearly there has been some control of the tumor by hormonal methods, but obviously the metastases are less susceptible to the control procedures than the primary tumor. We are of the opinion that adrenalectomy exerts more profound effects in this situation than in category *a* where there had been no retardation originally as a result of hormonal modification. However, as has been demonstrated, it was occasionally possible to induce regression of previously resistant primary prostatic cancer tissue.

Wilkins *et al.* (35) have shown that cortisone causes a decrease in the excretion of both 17-ketosteroids and "comb-growth" androgens in the urine of children with congenital adrenal hyperplasia. Our observations revealed that, although cortisone acetate produced a sense of euphoria, it did not significantly modify the course of prostatic cancer. It has been established that

the administration of cortisone acetate to the dog (5) is not followed by androgenic effects.

SUMMARY

Simultaneous bilateral adrenalectomy in man can now be done with comparative safety (29 consecutive cases without fatality.) The adrenalless man on adequate hormonal substitution therapy presents the metabolic picture of excellent health, although adrenal insufficiency can develop rapidly when hormonal replacement is inadequate to meet the demands of the organism.

In four cases of advanced cancer of the prostate which became reactivated after previous antiandrogen control, some or all of the following effects were observed: relief of intractable bone pain, gain in body weight, reduction of considerably increased acid phosphatase levels and of the least percentage of thermocoagulable proteins, together with an increase of total protein content of serum, increased hemoglobin and erythrocyte content of whole blood, and shrinkage of the primary tumor. Two cases of prostatic cancer did not improve to any significant degree after adrenalectomy. The period of observation of these cases has been 4–9 months.

Of six cases of advanced mammary cancer with metastases, two cases were improved, one patient was moderately benefited, and there was no demonstrable evidence of regression in three cases.

In four advanced neoplasms, other than prostatic or mammary, adrenalectomy caused no detectable regression of the tumor.

CONCLUSION

Adrenalectomy with maintenance of the patient on cortisone acetate can cause some regression of far advanced mammary and prostatic cancers for which there has been no previous therapy available.

PROTOCOLS

PROSTATIC CANCER

Case 1.—E.S., age 63, atrophy of primary prostatic cancer with active osseous metastases. Orchiectomy May, 1947, with recurrence of pain in back and rectum in 1950. Complete relief of pain after adrenalectomy in February, 1951. Urinary retention October, 1951, treated by total perineal prostatectomy; no cytologic evidence of active cancer cells in excised prostate. Gained 8.4 kg. in 251 days.

Case 2.—C.F., age 47, extensive neoplastic involvement of prostate, bones, and lymph nodes. Orchiectomy, 1948. On admission, March, 1951, moribund with massive edema of both legs and external genitalia; large constricting peri-rectal and abdominal masses; uncontrollable bone pain; bed-ridden and emaciated. After adrenalectomy cessation of pain; shrinkage of neoplastic masses. Gained 16 kg. in 230 days.

Case 3.—J.S., age 59. Died 72 hours after operation.

Case 4.—L.S., age 62, active primary tumor and metastases. Orchiectomy, 1946. For 2 years severe pain in legs requiring narcotics; urinary frequency and retention; large indurated neoplastic mass in seminal vesicle; extensive osseous metastasis. Following adrenalectomy no decrease in urinary retention; slight decrease in size of neoplastic mass; complete relief of pain. Died of pulmonary edema 49 days after adrenalectomy.

Case 5.—W.M., age 62, active primary prostatic neoplasm and osseous metastasis. Severe pain in back persisted after orchiectomy 60 days before. Prostate enlarged and indurated. Following adrenalectomy disappearance of pain, complete regression of prostatic enlargement as determined by palpation. Gained 13 kg. in 180 days.

Case 6.—J.F., age 59, active primary prostatic cancer with active osseous metastases. Orchiectomy April 3, 1950. In December, 1950, recurrence of severe sciatica poorly controlled by narcotics; osseous metastasis; prostate enlarged ++ and indurated; bed-ridden. Following adrenalectomy there was complete relief of pain and regression of the prostate to atrophic condition as determined by palpation. Returned to work. Gained 4 kg. in 158 days.

Case 7.—G.H., age 53, active primary prostatic cancer and osseous metastasis. Severe pain in shoulders, ribs, pelvis, and legs. Walnut-sized, hard nodule in prostate. No significant modification of neoplastic disease by adrenalectomy. Gained 4 kg. in 137 days.

MAMMARY CANCER

Case 8.—L.C., female, age 41. Died 48 hours after adrenalectomy.

Case 9.—M.B., female, age 47, extensive osteolytic metastases to pelvis and lymphedema of arm. Radical mastectomy in 1949; severe pain in pelvis and leg with osseous metastasis, November, 1950; rapid progression of the osteolytic lesion despite treatment with testosterone propionate and irradiation of pelvis. Following adrenalectomy partial relief of pain but continued advance of osseous lesions. Ovariectomy 56 days after adrenalectomy. Slight sclerosis of osteolytic lesions and considerable but not complete relief of pain. Gained 5 kg. in 168 days.

Case 10.—R.B., female, age 57, pulmonary metastases with dyspnea. No clinical improvement after adrenalectomy. Died 23 days after adrenalectomy.

Case 11.—J.B., female, age 40, large ulcerating mammary lesions with lymphedema of arm. Adrenalectomy followed by rapid advance of ulcerations.

Case 12.—G.N., female, age 43, hemothorax and metastasis to spine and pelvis. Radical mastectomy, 1945. Pain in spine with collapse of T_{11} vertebra and extensive destructive lesions in pelvis in June, 1950. Treated with testosterone propionate with complete relief of symptoms until May, 1951, when massive accumulation of fluid in pleural cavity occurred requiring repeated thoracentesis. Right adrenalectomy and bilateral ovariectomy, 6/13/51; left adrenalectomy, 6/28/51. No recurrence of pleural fluid of magnitude sufficient to require tapping in 155 days (Figs. 7, 8). Complete relief of symptoms.

Case 13.—N.C., male, age 53, intracranial and pulmonary metastasis. Radical mastectomy, May, 1948. In June, 1950, metastases found in routine chest roentgenogram. Bilateral orchiectomy, 11/25/50, followed by further progression of pulmonary lesions. For 3 months, anorexia and vomiting; for 3 weeks, diplopia. Adrenalectomy was followed by disappearance of neurologic signs and double vision. Regression of pulmonary lesions on x-ray examination 39 days (Figs. 1, 2) after operation. Gained 10 kg. in 117 days.

Case 14.—L.M., female, age 65. Carcinoma of right breast with cutaneous metastases in scar of left radical mastectomy (12/2/48). A large indurated mass was found in the right breast with extensive, red infiltrations in mastectomy scar. Within

4 weeks after adrenalectomy flattening and pallor of cutaneous lesion (Figs. 3, 4) and decrease in size of unoperated tumor in right breast.

Miscellaneous Cancers

Case 15.—A.K., male, age 58. An extensive squamous carcinoma of the urethra with metastasis to lymph glands of both inguinal regions and the right tibia was not benefited by bilateral adrenalectomy. Died 93 days after operation.

Case 16.—M.S., female, age 53. A very widely spread melanosarcoma with extensive pulmonary metastases was not improved after adrenalectomy. A great increase in size of pulmonary metastases was observed in roentgenograms made 4 weeks after operation.

Case 17.—J.S., male, age 41. Very large bilateral pulmonary metastases from a chorionic epithelioma of the testis was not affected favorably by bilateral adrenalectomy within 4 weeks after adrenalectomy.

Case 18.—M.N., female, age 22. An extensive undifferentiated carcinoma involving the right lung and left cervical lymph nodes continued to grow rapidly 4 weeks after adrenalectomy.

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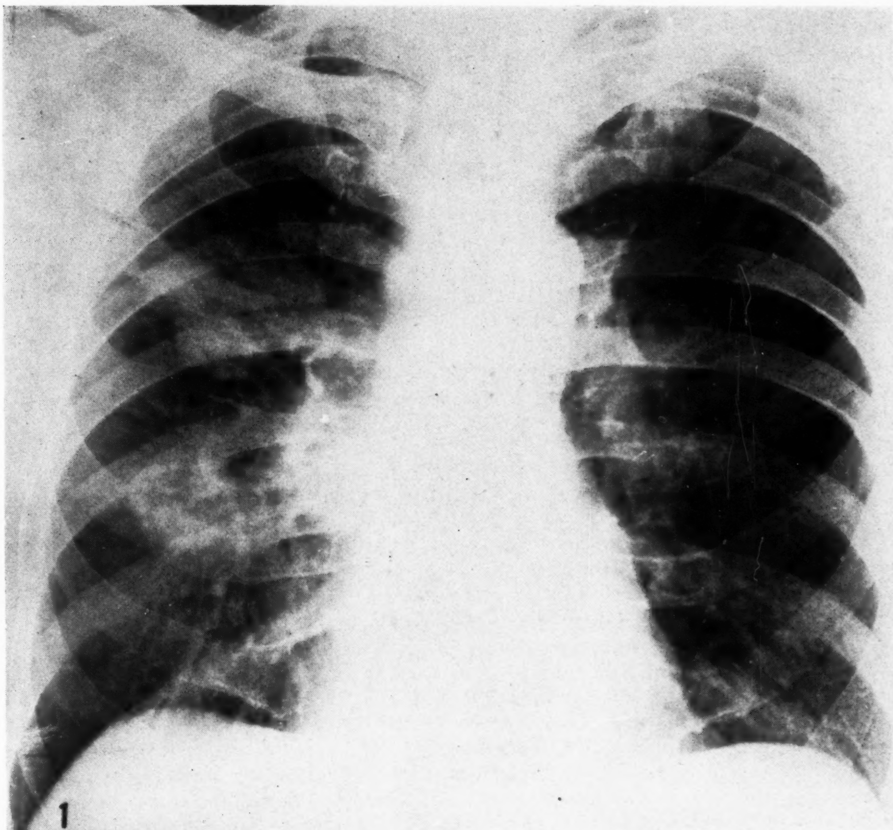

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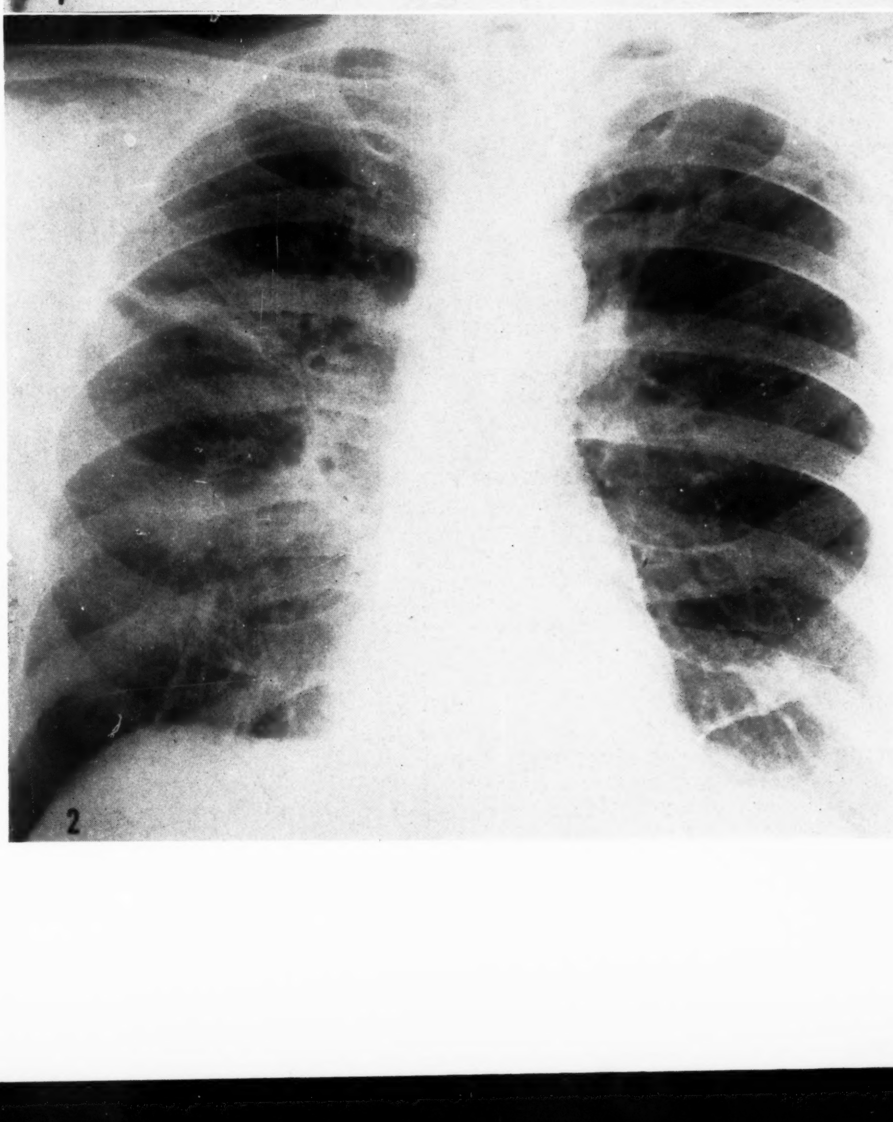

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FIG. 1.—Metastases to both lungs from mammary cancer in a male (Case 13) before adrenalectomy.

FIG. 2.—Roentgenogram of chest of a male with mammary cancer (Case 13) 39 days after adrenalectomy.

FIG. 3.—Recurrent carcinoma in a mastectomy scar in a woman (Case 14) before adrenalectomy.

FIG. 4.—Appearance of mastectomy scar (Case 14) 40 days after adrenalectomy.

FIG. 5.—Biopsy from dermal plaque of recurrent mammary cancer in a mastectomy scar (cf. Fig. 3) showing adenocarcinoma. (Dr. E. M. Humphreys.) ×475.

FIG. 6.—Biopsy from dermal plaque of recurrent mammary cancer in a mastectomy scar (cf. Fig. 4) 35 days after adrenalectomy showing an abundance of cancer cells which are shrunken with darkly stained nuclei. (Dr. E. M. Humphreys.) ×475.


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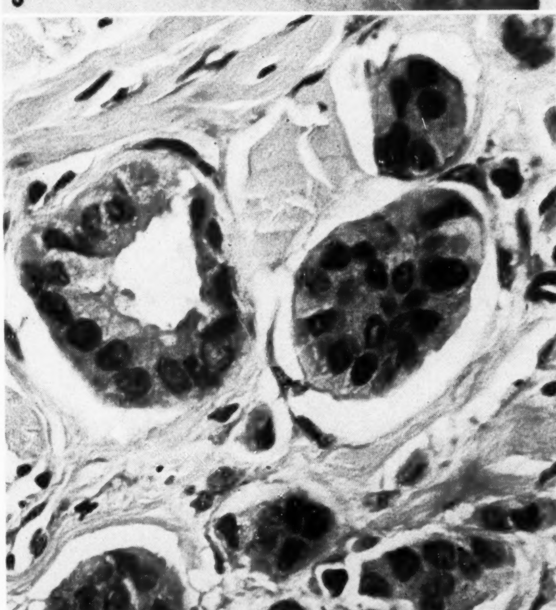
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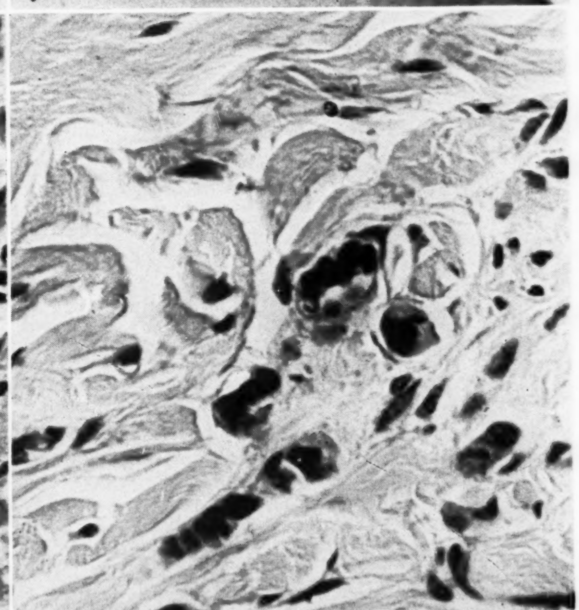
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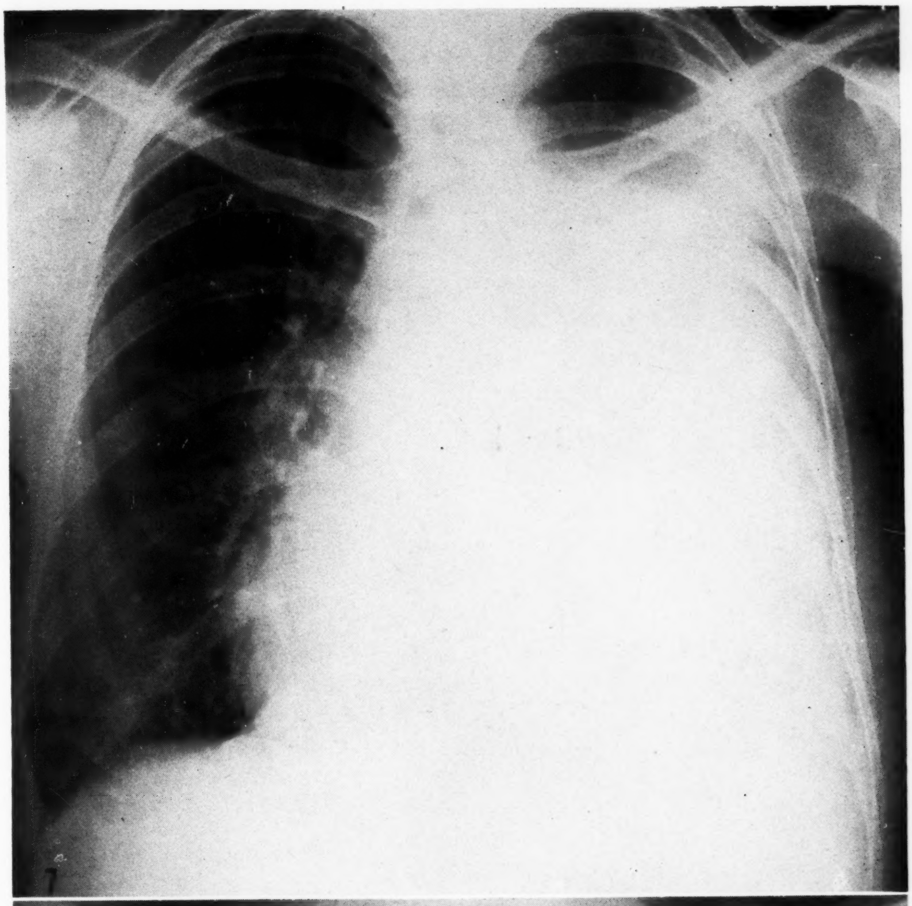
Fig. 7.—Metastatic carcinoma of the breast causing hemothorax in a woman (Case 12) before adrenalectomy.

Fig. 8.—Roentgenogram of the chest of a woman with metastatic mammary cancer (Case 12) 116 days after adrenalectomy. Thoracentesis has not been done since adrenalectomy.



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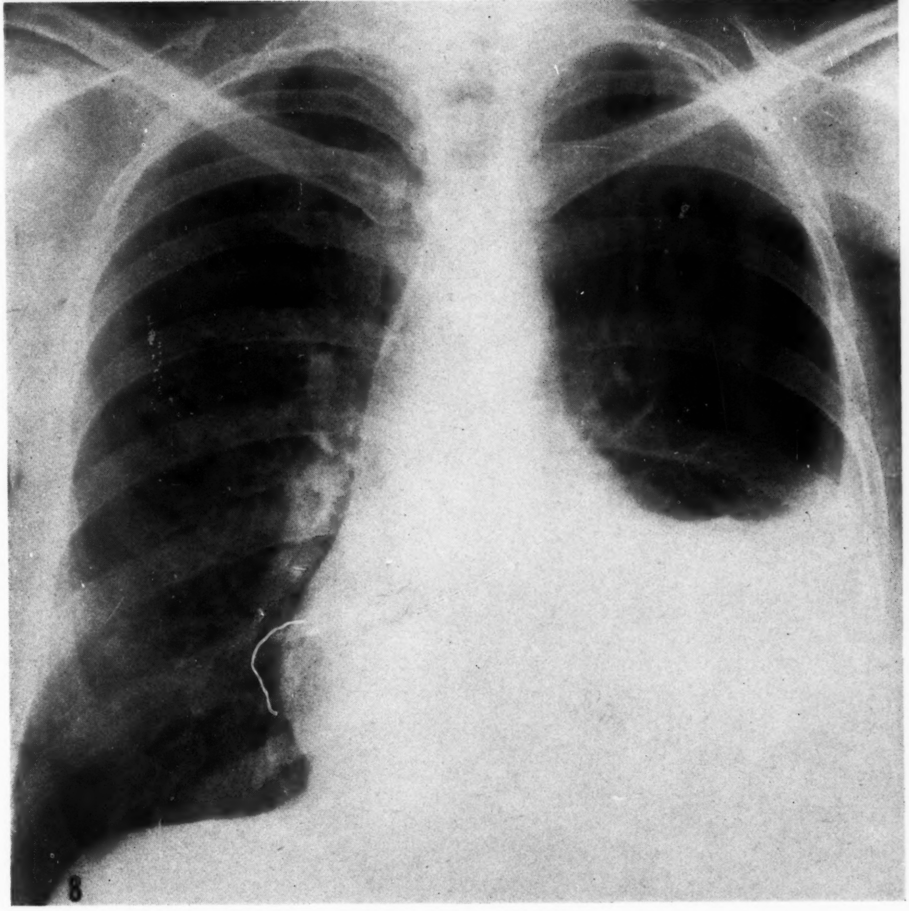

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Adrenal Tumors and Other Pathological Changes in Reciprocal Crosses in Mice

I. Strain DBA × Strain CE and the Reciprocal*

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INTRODUCTION

There has been a great variation in adrenal cortical growth and differentiation following early gonadectomy of certain inbred strains of mice. JAX strain DBA has been characterized by the occurrence of nodular hyperplasia of the adrenal cortex (9, 16–18), and JAX strain CE by adrenal cortical carcinoma (19–22). It had also been observed that, following early gonadectomy, such organs as the submaxillary gland, uterus, vagina, and others varied in extent and type of growth, depending upon the strain involved. Since these differences occurred generation after generation following gonadectomy, under uniform environmental conditions, it was believed that a genetic analysis of the factors responsible for the consistent appearances of these strain differences would be of value. The method employed was the study of all the named organs of F_1 offspring, both gonadectomized and intact, of reciprocal crosses of several inbred strains. This is part of such a study. Since critical ages for classification of tumors had not been established, the data were gathered at intervals throughout life of the animals.

Detailed observations on the uterus and the pituitary have been discussed in other reports (4, 7, 8, 24).

MATERIALS AND METHODS

Parental mice were Jackson Laboratory inbred strain DBA/2Wy and strain CE/Wy mice. Female DBA mice were mated to CE male mice (DBA× CE), and female CE mice were mated to male DBA mice (CE×DBA). The F_1 generation of approximately 150 animals was divided into two experimental and two control groups for each sex and for each reciprocal cross. Experimental animals were gonadectomized at birth and allowed to mature and age without mating and without further experimental treatment. Control mice were similarly observed without the operation and were maintained without mating. Animals from both experimental and control groups were autopsied at 15 days of age and at monthly intervals through life. Body weight was taken at autopsy. Gross and histologic observations were made on the endocrine glands and accessory reproductive organs for comparative studies. The tissues of various glands were fixed in modified Tellyesniczky's fluid and cut at 8 μ except for the pituitary; when the latter was abnormal it was fixed in modified Zenker's, serially sectioned at 4 μ, and differentially stained. Adrenal glands were serially sectioned unless greatly enlarged with tumor. Left ovaries of all control females were serially sectioned. The uterus and vagina and seminal vesicles were studied in cross section. Most of the tissues were stained with hematoxylin and aqueous eosin.

The skins of all animals were saved so that the mammary glands could be studied. All microscopic measurements were made with an ocular micrometer. Measurements proportional to the volume of the adrenals and ovaries were obtained by taking the length × width × depth of the gland and of the medulla. The significance of the mean of the various measurements has been determined by Fisher's (12) method for small samples using the available paired observations.

OBSERVATIONS

Adrenals: Controls

The adrenals of the control F_1 females of both reciprocal crosses were full sized by 5 months of age and remained so throughout life. Using the

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25 possible paired observations, it was found that the total adrenal size of the CE×DBA females was greater than for the DBA×CE. The difference was statistically significant. Mean difference = 0.5, $t = 2.1$, $P > 0.05$. Although both the cortex and the medulla were larger, the latter differences, when taken alone, were not significant. The x-zone was present but narrow at 15 days. It became wide at 1, 2, and 3 months and had disappeared by 5–6 months of age. Degeneration of the x-zone was somewhat earlier than in strain DBA (6) and similar to that in strain CE (19). Subcapsular "A" cells (19) were present and were focused in small areas by 3 months of age. There were similar cells in heavy bands and nodules under the capsule of the adrenal in the older age animals. A tumor, presumably originating from these cells, and somewhat larger than the space occupied by a normal adrenal was present in one adrenal of a 20-month CE×DBA F_1 animal (Fig. 4) and another smaller tumor in a CE×DBA F_1 animal at 22 months. An area where growth occurred in cells presumably related to the zona fasciculata was found in an F_1 CE×DBA animal 22 months of age. Although almost entirely within the confines of the cortex it compressed the adjacent zona fasciculata and medullary cells. (Hereafter nodules of this type will be called FN, referring to "Fasciculata nodule.") No enlarged "B" (19) cells were found.

The medullas were found to be consistently larger in the CE×DBA F_1 intact females than in the reciprocal cross (Tables 1 and 3). One medullary tumor (Fig. 7), an adenoma, was found in one 20-month-old CE×DBA F_1.

The adrenals of the control F_1 males, as with the females, were full size by 5 months of age and did not become appreciably smaller in old age (Tables 5 and 7). The male adrenals were, however, consistently smaller than those of females. Using the 27 possible paired observations it was found that the total adrenal size of CE×DBA normal males was larger than for the DBA×CE males. The reciprocal difference is in the same direction as with the females and again is statistically significant. Mean difference = 0.53, $t = 3.33$, $P > 0.01$. The cortex and the medulla of the CE×DBA were each larger, the former significantly so when considered alone. Mean difference = 0.4, $t = 3.6$, $P > 0.01$. The medullary difference did not prove to be statistically significant. The x-zone was present at 15 days, thin and hemorrhagic at 2 months, and absent at 3 months. "A" cells first appeared in small focal areas at 3 months in DBA×CE F_1 and at 4 months in CE×DBA F_1.

TABLE 1

OBSERVATIONS ON DBA ♀ × CE ♂ F_1 CONTROL FEMALES

Age (mo.)	Av. adrenal size (c. mm.)	Av. cortex size (c. mm.)	Av. medulla size (c. mm.)	Sub-maxillary gland (μ cell height)	Thyroid (μ cell height)	Body wt. (gm.)	Total uterus diameter (mm.)	Vagina cycle stage	Mammary gland growth	Ovary size (c. mm.)
0.5	0.9	0.8	0.1	11.00	8.20	8	0.4			1.3
1	2.5	2.2	0.3	12.42	10.00	15	1.3	proestrus	+	1.7
2	3.5	3.2	0.3	13.83	8.70	21	1.6	estrus	++	4.5
3	3.2	2.8	0.4	14.00	8.90	26	1.6	postestrus	++	6.8
4	2.5	2.1	0.4	14.73	9.30	24	1.3	"	+++	11.0
5	3.8	3.3	0.5	13.98	10.70	28	1.0	diestrus	+++	11.6
6	3.9	3.5	0.4	13.50	8.33	31	1.5	postestrus	+++	11.1
7	3.5	3.1	0.4	13.40	8.66	29	1.5	"	+++	16.2
8	3.1	2.7	0.4	13.50	8.83	34	1.7	estrus	+++	17.0
9	4.1	3.7	0.4	15.16	11.00	29	1.7	proestrus	+++	20.5
10	3.7	3.3	0.4	13.16	8.66	30	1.8	"	+++	16.0
11	4.1	3.6	0.5	13.60	8.50	32	1.7	"	+++	4.6
12	4.1	3.6	0.5	14.66		32	2.1	diestrus	+++	6.0
13	3.6	3.2	0.4	14.50	7.66	30	2.8	proestrus	+++	10.6
14	3.5	3.1	0.4	13.33	8.00	33	2.1	postestrus	+++	21.7
15	3.8	3.3	0.5	14.60	9.66	32	2.9	proestrus	+++	28.4
16	4.3	3.8	0.5	14.33	10.00	29	4.1	"	+++	61.2
17	4.8	4.2	0.6	15.66	9.66	28	very large	"	+++ tu.	47.4
18	4.4	3.9	0.5	13.73		30	2.6	metestrus	+++	23.2
19	4.4	3.9	0.5	13.66	9.33	33	5.6	estrus	+++ tu.	48.6
20	4.5	3.9	0.6	15.60	8.16	28	3.4	postestrus	+++	41.0
21										
22	3.5	3.1	0.4	13.40	10.16	28	1.7	postestrus	+++ tu.	28.5
23	2.2	1.8	0.4	12.93	7.16	33	1.9	diestrus	+++ tu.	10.8
24										
25	2.3	1.9	0.4	14.40	11.00	30	3.0	estrus	+++ tu.	37.9
26	2.3	1.8	0.5	14.83	9.16	31	2.2	diestrus	++ tu.	5.8
27										
28	2.5	1.9	0.6		7.50	32	2.3		+++	26.6

TABLE 2

OBSERVATIONS ON DBA ♀ × CE ♂ F_1 GONADECTOMIZED FEMALES

Age (mo.)	Left	Av. adrenal size (c. mm.)	Right	Av. medulla size (c. mm.)	Nodular hyperplasia	Adrenal tumor	Submaxillary gland	[Thyroid (μ cell height)	Body] wt. (gm.)	Uterus total diam. (mm.)	Vagina cycle stage	Mammary gland growth	Pituitary tumor
0.5		0.7		0.2	0	0 0	11.70	10.20	6	0.4			
1		1.9		0.3	0	0 0	13.30	10.40	13	0.4	diestrus		
2		2.0		0.4	0	0 0	14.10	10.50	22.5		"		
3		3.0		0.5	+	0 0	14.20	8.30	27	0.6	"		
4		3.8		0.5	+	0 0	14.12	8.40	30	0.4	"		
5		3.9		0.6	+	0 0	18.03		33.5	0.5	"		
6		3.3		0.4	+	0 0	18.06			0.4	"		
7		4.4		0.7	+	0 0	17.40	10.50	40	0.7	proestrus	+	
8		5.9		0.8	+	0 0	18.06	9.00	48	1.6	metestrus	+++	
9		5.6		0.5	+	+ +	18.83	10.33	34	1.8	"	++	
10		5.4		0.6	+	+ +	18.16	10.33	43		"	+++	
11	7.3	41.0	74.8	0.6	+	+ +	18.16	9.16	37	2.4	"	+++	
12	12.8	10.3	7.7	0.9	+	+ +	17.40	7.50	42	1.8	postestrus	+++	+?
13	4.7	4.8	4.9	0.7	+	+ 0	18.40	6.66	38	0.3	proestrus	−−−	0
14	216.0	220.5	225.3		+	+ +	18.73	9.83	38	2.3	postestrus	+++	0
15	12.2	216.5	421.9		+	+ +	19.26	12.16	38	1.8	"	+++	0
16	7.4	1984.1	3960.9		+	+ +	19.83	9.33	38	1.5	proestrus	+++ tu.	0
17	6.5	6159.9	12313.4		+	+ +	22.16	10.66	37.5	1.7	diestrus	+++	0
18	4.2	5514.6	11025.0		+	+ +	20.83	11.00	41	1.6	"	+++ tu.	+
19	14.6	2140.1	4265.6		+	+ +	18.83	7.16	32	2.3	postestrus	+++	+?
20	3.3	2227.3	4451.2		+	+ +	21.33	6.33	34	1.8	diestrus	+++	+
21	1600.0	8062.5	125.0		+	+ +	21.83	9.00	46	1.7	"	++ tu.	+

TABLE 3

OBSERVATIONS ON CE ♀ × DBA ♂ F_1 CONTROL FEMALES

Age (mo.)	Av. adrenal size (c. mm.)	Av. cortex size (c. mm.)	Av. medulla size (c. mm.)	Submaxillary gland (μ cell height)	Thyroid (μ cell height)	Body wt. (gm.)	Total uterus diameter (mm.)	Vagina cycle stage	Mammary gland growth	Ovary size (c. mm.)
0.5	0.7	0.5	0.2	12.10	9.10	7				1.2
1	1.9	1.6	0.3	12.72	8.20	16	0.5	proestrus	+	1.9
2	3.4	2.9	0.5	12.83	10.50	16	0.9	estrus	++	7.3
3	3.2	2.8	0.4	13.06	9.80	24	2.5	metestrus	++	4.9
4	3.0	2.6	0.4	14.45	10.60	22	2.0	proestrus	+++	3.8
5	3.1	2.6	0.5	14.03	10.00	26	1.8	diestrus	+++	9.7
6	2.8	2.4	0.4	15.60		28	1.3	"	++	9.0
7	2.8	2.4	0.4	14.66		30	3.2	proestrus	+++	
8	3.6	3.1	0.5	17.40	9.33	28	2.9	estrus	++	11.6
9	4.4	3.8	0.6	16.16	8.00	31	1.8	postestrus	+++	14.0
10	3.8	3.2	0.6	15.16	9.33	30	2.3	"	+++	19.6
11	4.6	3.9	0.7	15.60	6.33	34	2.1	estrus	+++	11.8
12	5.0	4.3	0.7	15.73	9.66	46	3.6	"	+++	34.4
13	4.0	3.3	0.7	15.40	7.00	28	2.0	postestrus	+++	16.8
14	4.0	3.4	0.6	♀ like	9.83	34	normal		+++	8.2
15	4.9	4.2	0.7	15.26	10.33	28	3.0	estrus	+++	18.9
16	4.1	3.1	1.0	14.26	7.83	27	3.0	postestrus	+++	22.1
17	4.4	3.7	0.7	14.83	8.00	28	5.7	diestrus	+++	
18	4.2	3.5	0.7	15.16	6.33	29	4.4	proestrus	+++	15.3
19	5.2	4.2	1.0	15.26	6.50	29	1.7	postestrus	+++	21.6
20	9.0	7.9	1.1	15.26	9.66	26	1.8	diestrus	++	13.3
21	4.5	3.6	0.9	14.93	6.33	29	very large	proestrus	+++	39.5
22	5.0	4.1	0.9	15.40	7.66	33	3.4	postestrus	++	48.2
23	3.7	2.9	0.8	13.40	6.16	32	1.7	diestrus	+++	8.0
24	3.4	2.7	0.7	13.33	7.00	30	2.3	postestrus	++	4.2
25	4.9	3.9	1.0	13.16	6.16	30	1.8	proestrus	++	13.3
26	3.6	2.8	0.8	13.06	6.66	25	3.2	"	++	15.6
27	5.0	4.1	0.9	12.33	7.00	29	2.8	"	+++	32.8

These cells were not so prominent in the males as in the females. Some nodules, formed from cells similar to the "A" cells, were present at 21 months of age and older. In a 20- and in a 21-month CE×DBA F_1, a partially hyalinized FN was present (Figs. 5 and 6). Nodular hyperplasia was present and extensive in a 21-month DBA×CE. It was also present in a DBA×CE at 22 months and a CE×DBA at 24 months. These nodules were thus more extensive in males than in females (Fig. 3). Medullary size was similar in the males of both crosses, in this respect differing slightly from the females. Occasionally, in both intact females and males of old age, areas of juxta-medullary hyalinization and degeneration of the cortex were observed (Fig. 8). In a few animals of old age, unusual and distinct bands of connective tissue were observed surrounding the medulla as if this tissue had undergone slight overgrowth.

ADRENALS: CASTRATE

The adrenals of the castrate females of both crosses will be discussed together as far as possible (Tables 2, 4, 6, and 8). Their similarities seemed to outweigh their differences as far as total size, time of appearance of tumor, and type of tumor. By 4 months of age the adrenals of the gonadectomized animals were larger than those of the controls. They attained a very large size by 12 months of age and older. The largest adrenal was 16,000 c. mm. or 2×2×4 cm. In all cases after 13 months of age, and in some cases before this age, there were tumors which were bilateral. In no case was there a markedly atrophic adrenal on the side opposite to a large-sized adrenal tumor. The CE×DBA animals died before 25 months of age, and the DBA×CE females died before 22 months of age; both died earlier than the controls, which lived to an average of about 28 months of age. Death in the gonadectomized mice was undoubtedly hastened by adrenal tumor development. Adrenal size corresponded more closely to the CE strain type than to the DBA strain type during the tumor period. It will be recalled that gonadectomized DBA female adrenals averaged close to 4 c. mm., with nodular hyperplasia in older ages. Gonadectomized CE adrenals with tumors were often 1–1.5 cm. in diameter.

TABLE 4

OBSERVATIONS ON CE ♀ × DBA ♂ F_1 GONADECTOMIZED FEMALES

Age (mo.)	Left	Av. adrenal size (c. mm.)	Right	Av. medulla size (c. mm.)	Nodular hyperplasia	Adrenal tumor	Submaxillary gland (μ cell height)	Thyroid (μ cell height)	Body wt. (gm.)	Uterus total diam. (mm.)	Vagina cycle stage	Mammary gland growth	Pituitary tumor
0.5		0.5		0.1	0	0 0	12.90	9.70	3.5	0.6	diestrus		
1		2.1		0.3	0	0 0	12.80	9.40	16	0.5			
2		3.0		0.4	0	0 0	14.40	7.70	17.5	1.2	postestrus		
3		2.8		0.4	+	0 0	15.70	10.20	32	0.3	diestrus		
4		3.2		0.5	+	0 0	14.28	7.90	32	0.3	"		
5		3.8		0.5	+	0 0	17.22	9.10		0.9	estrus		
6		5.4		0.7	+	+ +	17.60			0.6	proestrus	++	
7		4.1		0.5	+	0 0	18.00		36	1.3	metestrus	+	
8		6.1		0.5	+	+ +	19.00	9.00	36	1.1	"	+++	
9		6.2		0.5	+	+ +	18.83	9.50	29	norm	"	++	
10		4.7		0.6	+	+ +	18.06	7.16	44	2.0	"	++	
11		7.2		0.6	+	+ +	18.93	8.50	40	norm	"	+++	
12	729.0	378.0	27.0		+	+ +	19.00	10.66	27	1.6	"	+++	
13	10.3	19.7	29.9		+	+ +	15.93	9.83	28	1.6	postestrus	+++	+
14	10.8	1,267.8	2,524.8		+	+ +	18.00	9.00	38	1.8	"	+++	0
15	27.6	18.1	8.7		+	+ +	16.26	7.00	33	norm	"	+++	+
16	12.3	22.6	33.0		+	+ +	17.16	7.00	32	1.5	"	+++	+
17	51.8	31.9	12.1		+	+ +	18.33	10.83	32	1.5	"	+++	+
18	684.0	2,573.2	4,462.5		+	+ +	20.26	9.66	40	1.8	diestrus	+++	+
19	14.5	1,177.3	2,340.0		+	+ +	18.60	8.33	33	1.8	postestrus	+++	+
20	2,250.0	1,875.0	1,500.0		+	+ +	19.40	6.83	36	1.4	diestrus	+++	+
21	1,520.9	4,325.4	7,130.0		−	+ +	18.93	9.33	38	1.8	"	+++	+
22	161.1	1,633.4	1,605.8		−	+ +	19.66	10.16	40	1.7	"	+++	+
23	6,480.0	8,280.0	10,008.0		+	+ +	19.93	8.66	41	2.1	"	+++	+
24	1,422.9	7,146.5	64.0		−	+ +	21.16	7.00	38	1.9	"	+++	0

The total adrenal size of the gonadectomized F_1 females was larger in the DBA×CE series than in the CE×DBA series, but not significantly so. However, considering the period before large adrenal cortical tumors developed, i.e., through 15 months, the total adrenal size of the CE × DBA females was significantly larger than the DBA×CE; mean difference=75.6, t = 4.5, P > 0.001. The reciprocal size differential is in the same direction as that for the virgin control females and normal males. In this series it was not possible to treat the cortex and the medulla individually, since the

TABLE 5

OBSERVATIONS ON DBA ♀ × CE ♂ F_1 CONTROL MALES

Age (mo.)	Av. adrenal size (c. mm.)	Av. cortex size (c. mm.)	Av. medulla size (c. mm.)	Nodular hyper-plasia	Sub-maxillary gland (μ cell height)	Thyroid (μ cell height)	Body wt. (gm.)	Vesicular gland*	Meta-plasia anterior prostate	Mam-mary gland growth*
0.5	1.0	0.8	0.2	0	11.60	9.70	8	—	—	R
1	1.5	1.2	0.3	0	14.28	10.00	15	S	0	R
2	2.7	2.1	0.6	0	22.72	8.20	23	+++	—	R
3	1.7	1.4	0.3	0	21.20	9.60	21	—	0	R
4	1.7	1.3	0.4	0	22.68	6.40	32	+++	0	—
5	2.6	2.1	0.5	0	22.18	9.20	36.5	—	0	R
6	2.2	1.8	0.4	0	21.66	5.66	29	+++	0	R
7	1.7	1.3	0.4	0	22.60	8.50	31	+++	—	R
8	1.9	1.3	0.6	0	22.40	8.33	27	+++	0	R
9	2.5	1.8	0.7	0	21.66	7.00	30	+++	0	R
10	1.6	1.2	0.4	0	22.16	8.33	33	+++	—	R
11	1.8	1.3	0.5	0	22.40	9.83	31	+++	—	—
12	2.2	1.5	0.7	0	22.00	9.50	33	+++	—	R
13	2.3	1.5	0.7	0	20.93	8.83	37	+++	—	—
14	2.6	1.8	0.8	0	18.66	9.16	35	+++	0	—
15	2.3	1.7	0.6	0	20.93	8.16	33	+++	0	R
16	2.6	1.7	0.9	0	21.26	6.83	34	+++	0	—
17	1.9	1.4	0.5	0	21.33	9.50	39	+++	0	—
18	2.2	1.8	0.6	0	20.66	6.66	36	+++	0	R
19	2.8	1.8	1.0	0	20.66	9.33	41	+++	0	R
20	2.1	1.4	0.7	0	20.33	9.16	32	+++	—	R
21	2.3	1.7	0.6	+	19.83	7.50	36	+++	—	R
22	2.2	1.6	0.6	+	19.83	8.66	34	+++	—	R
23	2.4	1.8	0.6	0	18.16	8.33	34	+++	—	R
24	1.8	1.2	0.6	0	18.50	9.16	30	++	—	R
25	2.1	1.5	0.6	+	17.60	9.83	30	++	—	R
26	3.5	2.7	0.8	+	19.33	8.83	30	++	—	—

* R = rudimentary; S = small.

TABLE 6

OBSERVATIONS ON DBA ♀ × CE ♂ F_1 GONADECTOMIZED MALES

Age (mo.)	Left	Av. adrenal size (c. mm.)	Right	Av. medulla size (c. mm.)	Nodular hyper-plasia	Adrenal tumor	Sub-maxillary gland (μ cell height)	Thyroid (μ cell height)	Body wt. (gm.)	Vesic-ular gland*	Meta-plasia anterior prostate	Mam-mary gland growth*	Pitui-tary tumor
0.5		0.6		0.1	0 0	0 0	11.00	8.50	5.5	—	—	—	
1		2.0		0.3	0 0	0 0	13.40	10.40	14.5	S	0	R	
2		2.4		0.4	0 0	0 0	12.50	8.80	21	—	0	R	
3		3.0		0.4	0 0	0 0	14.50		25	—	—	—	
4		3.3		0.5	+ 0	0 0	13.75	7.30	30.5	—	0	—	
5		2.9		0.5	+ +	0 0	14.68	9.50	38	S	0	—	
6		2.4		0.4	+ +	0 0	16.60		44	S	0	—	
7		2.6		0.4	+ +	0 0	16.60	6.66	45	S	0	—	
8		3.1		0.5	+ +	0 0	16.40	7.83	50	S	0	—	
9		4.2		0.5	+ +	0 0	17.66	9.83	41	+	0	—	
10		3.3		0.4	+ +	0 0	17.66	9.00	46	+	+	+	
11		3.9		0.4	+ +	0 0	17.26	8.33	42	+	+	+++	
12	3.9	4.2	4.5	0.6	+ +	+ +	17.06	10.83	43	+	+	++	
13	6.8	7.0	7.1	0.8	+ +	0 0	17.93	10.50	44	++	+	++	?
14	34.7	24.8	14.9		+ +	+ +	17.06	8.16	50	++	+	+++	
15	576.0	291.6	7.2		+ +	+ +	18.93	8.16	38	++	—	++	
16	4.4	19.8	35.2		+ +	+ +	18.33	8.50	36	++	—	+++	
17	99.0	52.4	5.7		+ +	+ +	17.06	9.33	37	+	+	+++	+
18	450.0	227.1	4.9		+ +	+ +	18.83	9.00	40	+	+	++	+
19	19.5	1,324.1	2,642.6		+ +	+ +	21.60	7.16	38	++	—	++	?
20	6.0	3,153.0	6,300.0		— —	+ +	19.60	10.83	38	+	+	+++ tu.	+
21	4.6	2,754.3	5,504.0		+ +	0 +	21.33	8.50	37	+++	0	+++	+
22	12.6	1,686.3	3,360.0		+ +	+ +	22.00	6.33	32	+++	+	+++ tu.	+
23	4.4	7,339.2	14,674.0		+ +	+ +	23.73	10.50	40	+++	+	++	—
24	4.5	4.3	4.1		+ +	+ +	16.73	7.66	30	++	—	++	—

* R = rudimentary; S = small.

TABLE 7

Observations on CE ♀ × DBA ♂ F_1 Control Males

Age (mo.)	Av. adrenal size (c. mm.)	Av. cortex size (c. mm.)	Av. medulla size (c. mm.)	Nodular hyper-plasia	Sub-maxillary gland (μ cell height)	Thyroid (μ cell height)	Body wt. (gm.)	Vesicular gland*	Meta-plasia anterior prostate	Mam-mary gland growth*
0.5	0.8	0.7	0.1		12.20	9.10	8	—	—	—
1	1.8	1.6	0.2		14.66	9.00	15	S	0	—
2	1.9	1.5	0.4		21.80	9.80	22	++	0	—
3	2.4	1.9	0.5		23.05	9.90	25	—	0	R
4	2.0	1.6	0.4		23.02	10.30	27	+++	0	R
5	2.2	1.7	0.5		22.66	10.50	30	+++	0	R
6	2.4	1.8	0.6		22.33	8.66	32	+++	0	R
7	2.6	2.1	0.5		21.93	9.33	33	+++	0	—
8	2.5	2.0	0.5		♂ like	8.66	34	+++	0	—
9	2.5	2.0	0.5		21.66	9.16	36	+++	0	R
10	3.4	2.6	0.8		20.83	7.83	32	+++	0	—
11	3.0	2.4	0.6		21.60	8.83	34	+++	0	—
12	2.8	2.1	0.7		♂ like	10.16	35	+++	—	R
13	3.2	2.3	0.9		21.50	8.00	31	+++	0	—
14	2.8	2.1	0.7		20.73	9.50	36	+++	0	R
15	3.0	2.2	0.8		20.83	8.16	34	+++	0	—
16	2.6	2.0	0.6		21.16	8.66	36	+++	0	R
17	3.7	2.9	0.8		20.83	8.50	34	+++	0	R
18	3.3	2.4	0.9		21.00	8.33	30	+++	0	—
19	3.3	2.4	0.9		20.83	7.16	33	+++	0	R
20	3.1	2.3	0.8		20.50	10.16	30	+++	0	R
21	2.8	2.0	0.8		20.50	10.33	30	+++	0	—
22	3.2	2.5	0.7		15.26	8.66	24	++	0	—
23	3.2	2.3	0.9		19.60	8.16	33	++	0	—
24	3.5	2.3	1.2	+	20.06	8.16	32	++	0	—
25	2.7	2.0	0.7	+	19.40	9.33	33	++	0	R
26	1.8	1.3	0.5	0	18.83	8.33	34	++	0	—
27	3.4	2.3	1.1	+	18.93	7.66	34	++	0	—

*R = rudimentary; S = small.

TABLE 8

Observations on CE ♀ × DBA ♂ F_1 Gonadectomized Males

Age (mo.)	Left	Av. adrenal size (c. mm.)	Right	Av. medulla size (c. mm.)	Nodu-lar hyper-plasia	Adrenal tumor	Sub-maxillary gland (μ cell height)	Thyroid (μ cell height	Body wt. (gm.)	Vesicular gland*	Meta-plasia anterior prostate	Mammary gland growth*	Pitui-tary tumor
0.5		1.0		0.2	0 0	0 0	12.60	9.70	4.5	S	—		
1		2.3		0.3	0 0	0 0	13.80	9.40	20.5	S	—		
2		3.4		0.4	0 0	0 0	14.50	7.70	21	S	—		
3		2.5		0.3	0 0	0 0	14.80	10.20	22	S	—		
4		3.2		0.5	+ 0	0 0	13.96	7.90	29.5	S	—		
5		3.6		0.5	+ +	0 0	15.73	9.10		S	0		
6		4.1		0.6	+ +	0 0	16.00			S	0	R	
7					+ +	0 0	15.60		35	S	0	R	
8		4.3		0.5	+ +	0 0	16.66	7.50	41	S	0	++	
9		3.7		0.5	+ +	0 0	16.93	8.33	38	+	0	+	
10		4.7		0.6	+ +	0 0	17.06	8.16	43	++	0	++	
11		5.0		0.5	+ +	0 0	17.00	9.83	34	+	0	++	
12	5.5	6.4	7.3	0.6	+ +	+ +	17.66	7.83	40	+	0?	++	
13	4.7	5.1	5.6	0.6	+ +	+ 0	18.00	8.00	30	+	—	++	
14	8.3	9.5	10.6	1.2	+ +	+ 0	17.66	10.50	29	++	+	++	
15	14.2	17.1	20.1	1.0	+ +	+ +	18.50	6.66	37	++	+	+++	?
16	38.1	22.9	7.7		+ +	+ +	18.50	9.00	31	++	+	+++	?
17	27.2	33.1	39.0		+ +	+ +	19.50	9.33	35	+++	+	+++	+
18	2,600.0	1,303.3	6.6		+ +	+ +	16.16	12.00	39	++	+	+++++	+
19	9,810.0	4,908.3	6.6		+ +	+ +	21.33	9.33	38	++	+	+++	+
20	19.0	504.5	990.0		+ +	+ +	16.33	4.66	32	+	+	+++	+
21	8,140.0	4,114.0	88.0		+ +	+ +	20.26	6.33	39	++	+	++	+?
22	6,851.2	3,432.4	13.5		+ +	+ +	22.00	9.00	40	+++	+	++	
23	6.6	1,620.3	3,234.0		+ +	+ +	20.50	9.66	28	++	+	+++	+
24	480.0	2,832.0	5,184.0		+ +	+ +	20.66	6.33	36	++	—	+++	+
25	648.0	4,248.0	7,920.0		+ +	+ +	17.33	9.16	46	++	+	+++	+
26	7,560.0	3,783.6	7.2		+ +	+ +	18.60	8.50	38	++	—	++	+?

*R = rudimentary; S = small.

cortical tumors caused the medullary cells to be distributed in an atypical manner, especially after 8–10 months of age.

"A" cells were present in distinct focal areas at 2 months of age, and "B" cells appeared at 3 months of age. None of the latter were found in the controls, except in old age. The areas formed by these cells rapidly increased in size, penetrating to the medulla, in some instances, by 4 months of age. The x-zone was degenerating under focal areas of "A" cells at 2 months of age and had disappeared entirely at 6 months of age.

The first nodule of the type found to enlarge progressively to carcinoma in strain CE appeared in a CE×DBA F_1 animal at 6 months of age (Table 4). Such nodules did not appear in DBA×CE females until 9 months of age (Table 2). There appears to be a definite difference, in this respect, between the reciprocal F_1 generations.

Variations in cortical carcinoma morphology have previously been described (10). Again the presence of areas in tumors that resembled seminiferous tubules of cryptorchid testis and contained cells similar to Sertoli cells were observed (Fig. 1). These were usually found in the smaller of the two bilateral tumors, possibly because of the fact that they were serially sectioned, or possibly because of a modification caused by the opposite large adrenal tumor. Another abnormality noted in a DBA×CE F_1 at 19 months of age was an attempt at follicular arrangement similar to that found in the cortex of the ovary (Fig. 2).

There were no notable differences in the size or structure of the medulla during the first few months of life.

The adrenals of the young gonadectomized males were almost always larger than those of the controls. This was probably due to the presence of the larger female-like x-zone in early ages in the gonadectomized mice. The adrenal tumors of the females were almost invariably larger than those of the males, and as in strain CE the tumors in the females occurred earlier.

With the use of all the 24 possible paired observations, it was found that the total adrenal size of the gonadectomized CE×DBA males was larger than that of the gonadectomized DBA×CE animals. Mean difference = 803, $t = 2.3$, $P > 0.05$. The largest male adrenal tumor was in a DBA×CE F_1 male 23 months of age. It had a volume of 14,674 c. mm. or 2.9 × 2.3 × 2.2 cm.

"A" cells were present in focal areas at 2 months of age and "B" cells at 4 months of age, becoming extensive at 6 months. The x-zone had usually disappeared at 6 months of age, although portions of it could be observed as late as 9 months in some individuals. Nodules similar to the cortical carcinomas were first present at 12 months, fairly large at 14 months, and very large at 19 months and beyond that age.

Other Organs

Submaxillary.—The submaxillary glands of the gonadectomized mice respond to male type hormones by increase in size, change in color, and by histological changes toward the characteristic male type. In the present study the height of the cells of the terminal tubules was measured and the position of the nuclei within these cells noted. Increased height of the cells of the tubules seems to be a good measure of male-type hormone activity. The males have a cell height that ranges in mid-life between 20 and 23 μ, and the females between 13 and 16 μ, with CE×DBA F_1 females slightly higher than DBA×CE F_1 females. Using the 24 possible paired combinations, the mean difference was 0.71, $t = 2.67$, $P > 0.02$. In each sex the height is less in early ages and in extreme old age. The nuclei occupy a central position in these cells in females and a basal position in males. In the castrate females at 5 months and beyond, the tubule cells are taller than those of normal females and in some cases may attain the height found in normal males. The position of the nuclei, however, was not as uniformly basal as in normal males. Castrate DBA×CE F_1 females had slightly higher terminal tubule cells than did animals of the reciprocal cross. The reciprocal gonadectomized males were similar to one another.

Thyroid.—An attempt was made to determine thyroid activity by measuring cell height on twenty cells of various follicles in the thyroids of each animal. Cell height in the various groups ranged from 6 to 11 μ, and there were no striking differences between the gonadectomized and control animals. Although the difference between the cell height was statistically significant and larger for the control and gonadectomized DBA×CE than for their reciprocal groups ($P > 0.01$), it must be realized that the number of measurements per gland were small. A more intensive study of these differences is desirable. In general, CE×DBA F_1 mice had more stroma in later life and lower follicular cell height than did the DBA×CE F_1. This applied both to the gonadectomized and control female groups.

Ovary.—The left ovary of each animal was measured in a manner similar to that used for obtaining total adrenal size. No consistent size differences were established between the DBA×CE and CE×DBA F_1 animals. The ovaries were similar in size to the DBA ovaries (11) but were

even larger than those reported for DBA. One was 73 c. mm. in volume. The corpora lutea were retained, hyalinized, and in some cases calcified. A few granulosa-cell tumors, comparable to those found in strain CE, were observed in DBA×CE and CE×DBA, in older ages.

Testis.—A testicular tumor was observed in a 21-month DBA×CE. The slide was unfortunately lost, however, in the laboratory fire. In each cross a few spermatozoa were observed in the seminiferous tubules at 1 month and many in the tubules at 2 months and thereafter. Atrophic changes in the testes in both reciprocal hybrids were observed beginning at 13 months, and there was evidence that sperm production was considerably reduced after 20 months.

Vesicular glands and prostates.—No reciprocal differences were noted in the control animals. In the gonadectomized animals there was minor evidence of stimulation at between 6 and 10 months. At 10 months and in succeeding months there was squamous metaplasia of the prostatic epithelium. There was also some secretion in the vesicular glands and extensive hyalinization of stroma around the glands (3), the latter an indication of the presence of estrogen. The vesicular glands showed definite enlargement from 10 months of age on and became nearly normal in size, as compared to the controls. They were probably better maintained in old age than in the control animals.

Mammary glands.—An ourstanding difference was that all adult DBA×CE females had areas of overgrowth, so-called precancerous lesions, from 7 months on, and mammary gland tumors from 17 months on. There were no similar areas and no mammary tumors in the CE×DBA. This is a clear-cut reciprocal cross difference.

In the gonadectomized females stimulation of the mammary glands, as evidenced by lengthening and branching of the ducts, occurred by 6 months of age. End buds which were present from 1 to 3 months of age in control females occurred at 6 months in DBA×CE and 6–12 months of age in CE×DBA. Alveolar development was noted at about 11 months of age in both groups. As in the DBA×CE control females, there were areas of precancerous lesions and tumor in DBA×CE gonadectomized mice. Precancerous lesions were first noted at 9 months, and the first tumor at 16 months of age. In the CE × DBA females, as in the control CE × DBA, there were no areas of precancerous lesions and no tumors.

Mammary glands were rudimentary in all control males, but larger rudiments were found in DBA×CE than in CE×DBA. There was no evidence of end buds, alveolar growth, or the occurrence of mammary tumors in the control males.

In the gonadectomized CE×DBA males, duct growth was evident by 8 months and in the DBA×CE males by 10 months of age. In later life the glands of both F_1 hybrids approached the growth observed in virgin females. An outstanding difference between all males and females is that in females all five pairs of glands are typically present, but in males only a few glands may be clearly developed, and growth among these glands is more variable. End buds were found in CE×DBA from 9 to 13 months and in the DBA×CE at 10 and 11 months only. There was some alveolar formation at 11 months in DBA×CE and at 14 months in CE×DBA. After those ages alveolar development was present whenever a pituitary tumor was present. The outstanding difference in the two series of F_1's is the occurrence of areas of precancerous lesions from 15 months on in the DBA×CE and the occurrence of two mammary tumors in this series. There were no such developments in CE×DBA males.

Uterus.—Adult size of the uterus was attained by 2 months of age in the DBA×CE control females and by 4 months of age in the reciprocal cross. It is interesting to note that CE females are definitely late in maturing compared to DBA's, as based on time of first litter. The result observed in hybrids may therefore indicate maternal influence. The CE×DBA uteri were slightly larger than the DBA×CE. Mean difference = 0.3 mm., $t = 2.3$, $P > 0.05$. However, in many cases the measurements were not on an absolute cross section, and layers of the organ were missing. At 7 months of age in the CE×DBA females adenomyosis was noted. This first occurred in the DBA×CE females at 9 months. Cystic glandular hyperplasia was noted in both groups of control females at 8 months of age. Adenomatous hyperplasia occurred at 11 months. Details of these pathological changes are being reported elsewhere (4).

In the two gonadectomized series, adult uterine size was not attained until 7–8 months of age. Comparing the period 8 months of age and beyond, the uteri of the control females were significantly larger than those of the gonadectomized mice. Difficulties of measurement were again encountered. Cystic glandular hyperplasia was noted in both groups at 8–9 months, but adenomyosis did not appear until 14 months in the DBA×CE gonadectomized females and 16 months in the reciprocal group. Adenomatous hyperplasia occurred much later.

Vagina.—No attempt was made to determine the estrous cycle of any of these mice. Hence, mice

autopsied were at various stages of the cycle. In both groups of control females the vaginas were in proestrus at 1 month of age. There were no unusual changes in the vagina; in very old age, however, they were found to be more frequently in diestrus.

In the gonadectomized females of both crosses the vaginas were in a diestrous condition until 6 months in the CE×DBA females and until 7 months in the DBA×CE females. Resting stage or diestrus appeared and remained permanent at 20 months in both groups. Details of the condition of the vaginas of the animals are discussed along with the uterus in other reports (4, 5). CE × DBA gonadectomized females had a vaginal epithelium which showed stimulation at an earlier age and was more continuously thickened during mid-life. It exhibited a cornified layer for more months than the DBA×CE gonadectomized females.

Pituitary.—The hypophyses of all normal animals were examined, and few abnormalities were noted. The main types of anomaly that occurred were two types of colloid cysts. One type of cyst was lined by thin epithelial cells; these cysts were usually quite small. The other type was lined by cuboidal ciliated cells.

In the gonadectomized series of both reciprocal crosses after 13 months of age gross abnormalities, opaque nodules and hemorrhagic nodules, were found. Microscopic examination showed that the opaque areas consisted of stages of transitional cell types presumably leading to basophile adenomas, while hemorrhagic nodules had already become basophile adenomas. The physiological action of these adenomas was manifested in the mammary gland development. There seemed to be little reciprocal differences, with perhaps the exception of the gonadectomized females; fewer adenomas were noted in the DBA×CE F_1.

There were other abnormalities noted in the hypophyses of the gonadectomized groups. Cysts were present, as in the control groups and in a 26-month-old gonadectomized CE×DBA F_1 male there was a cholesteatoma and amyloid degeneration. The pituitary changes have been reported in detail elsewhere (7, 8, 24).

Body weight.—DBA×CE control males and females and DBA×CE gonadectomized males and females were found to be heavier than their reciprocals when total possible weight comparisons were summed. When the significance of the mean difference was determined, it was found that the differences for the control males were not significant and that the differences for the other three classes were statistically significant.

A summation of all possible body weight comparisons without regard to reciprocal differences also shows that the rank for body weight, from light to heavy, was as follows: female, male, gonadectomized female, and gonadectomized male.

Tumors of various sites.—In addition to the tumors already mentioned (adrenal, pituitary, mammary, ovarian), the following were observed. Gonadectomized DBA×CE F_1 females had two lymphoblastomas and one reticuloendothelioma. Gonadectomized CE×DBA F_1 females had one fibrosarcoma in the region of the vaginal opening. Control DBA×CE F_1 females had three lung adenomas, two lymphoblastomas, and one fibrosarcoma of the uterus. Control CE×DBA F_1 females had a lung adenoma, two lymphoblastomas, a fibrosarcoma of the uterus, a papillary cyst adenoma of the ovary, and a tumor (unclassified) of the vagina. In the gonadectomized DBA× CE F_1 males one lung tumor was found. The gonadectomized CE×DBA F_1 males had two lymphoblastomas and one lung adenoma. Control DBA×CE F_1 males had four lymphoblastomas and two lung tumors, while control CE×DBA F_1 males had a papilloma of an eyelid and one lung adenoma.

DISCUSSION

Since this is primarily a pilot experiment, the authors wish to point out that differences that have occurred in these series of animals are only suggestive, and for detailed analysis larger numbers of animals should be studied. A developmental approach was used, since the critical times for classifying for different hereditary, maternal, or endocrine differences were not known in advance.

The changes that occurred in the pituitary in conjunction with the adrenal tumors and which seem to have a counterpart in man seemed important enough for separate detailed presentation (8). The uterine abnormalities which were striking and could be studied on a developmental basis were also presented separately (5).

More study could not be given to a number of special tumors at various sites, because the tissues were lost in the Bar Harbor fire of 1947.

The ground work has now been laid for an analysis of reciprocal backcross groups which is now being carried on with larger numbers at certain periods with special reference to any part of the developmental period desired and more particularly to the relation to known genes. Some progress is being made in these studies.

In all, four main types of adrenal cortical abnormalities were found: (*a*) nodular hyperplasia, (*b*) adrenal cortical carcinoma, (*c*) tumors pre-

sumably of "A" cell origin, and (*d*) focal glomerulosa and fasciculata hypertrophy.

The changes in the adrenals in normal animals in old age are especially interesting. The large nodules, apparently of "A" cell origin, and the occurrence of "B" cells, similar to those which appear in nodular hyperplasia in old age, would perhaps indicate a condition similar to that reported for strain NH (14). These changes occurred earlier following gonadectomy in both strain NH animals and in these F_1 series (14). The occurrence of nodular hyperplasia in normal animals at a young age has generally been interpreted as a failure of gonad function, possibly involving high pituitary activity. In these series of animals the gonads may not have failed entirely in their function, but the adrenals have begun to assume morphologic changes definitely associated at an earlier age with hormone production in gonadectomized animals. This may, in turn, be reflected in the pathological changes in the uterus.

The occurrence of adrenal cortical carcinoma in the gonadectomized F_1 animals is similar to that of the parental strain CE and not to parental strain DBA. It is clear that it is not maternally influenced as is the mouse mammary tumor incidence, by a virus-like particle carried in the mother's milk (2, 13). There is, moreover, no strong evidence of any other maternal influence on the incidence of adrenal cortical carcinomas. On the basis of the F_1 data the inheritance of this type of tumor cannot be assigned to any one gene or to any one gene complex but can be considered definitely as an inheritance from strain CE. The sex difference in the time of appearance of the adrenal cortical carcinomas in both F_1's was the same as that observed in strain CE. CE adrenal tumors have been studied following transplantation (23), but it has not yet been possible to study the F_1 adrenal tumors after transplantation.

The submaxillary gland and various other organs furnish evidence that the inheritance from CE has influenced the potentials for hormonal secretion from the adrenal tumors more definitely than has strain DBA. Strain DBA produced changes associated with estrogenic secretions only, while strain CE and the present F_1 series produced evidence of androgenic as well as estrogenic secretion.

The incidence or nonincidence of so-called precancerous lesions in the mammary glands showed definite maternal (extra-chromosomal) inheritance in the reciprocal F_1 generations. Maternal influence on the type of mammary gland physiology has also been reported in a cross of DBA×A mice by Bittner (1).

The time of attainment of adult uterine size may also be subject to maternal influence. This opens up the interesting question of the genetic nature of differences in the rate of maturity in mammals, which is a field in need of further investigation.

Another possible maternal effect is seen in the time of appearance of the nodules leading to adrenal cortical carcinoma. Here the F_1 animals from CE mothers (the parent strain which forms these neoplasms following gonadectomy) showed these nodules distinctly earlier than F_1's derived from DBA mothers. Resistance to formation of this type of tumor introduced by DBA mothers is also indicated by smaller size of the tumors in DBA×CE F_1 animals.

An interesting reciprocal cross difference associated with sex was observed in the size of the medulla. The medullary size of CE×DBA females was consistently greater than that of DBA×CE females. The males of both crosses did not show these differences. This is an example of hormonal influence in the males equalizing a genetic (probably extra-chromosomal) difference which was evident in females.

There is some evidence that hybridization in itself brought about and emphasized pathological conditions not occurring extensively in the parental animals, such as basophilic tumors of the pituitary, uterine growth abnormalities, and changes in the adrenals of older intact animals. This may be related to the findings of Little that there was an increase in tumor types and in certain tumor frequencies in F_1 generation mice of crosses between *Mus musculus* and *Mus bactrianus* (15).

SUMMARY AND CONCLUSIONS

Approximately 150 intact and gonadectomized mice from reciprocal crosses of strain CE and DBA were autopsied and studied from 15 days to 24 months of age and beyond. Adrenal cortical tumors of the type found in strain CE and not of the type found in DBA occurred in the gonadectomized F_1 mice. Histological evidence of hormonal stimulation presumably from the adrenal tumors was found in the submaxillary gland and accessory reproductive organs and paralleled that observed in parent strain CE.

Changes that previously had not been seen extensively in either parental strain were pituitary adenomas, cystic glandular hyperplasia and adenomyosis of the uteri, and large nodules of "A" cells and some of "A" and "B" cells in adrenals of old intact animals.

Evidence of definite maternal influence was obtained for the occurrence of mammary tumors in

DBA×CE F_1 intact females, as well as in gonadectomized females and males of the same cross.

No outstanding evidence of maternal influence on the occurrence of adrenal cortical tumors or tumors at other sites was found.

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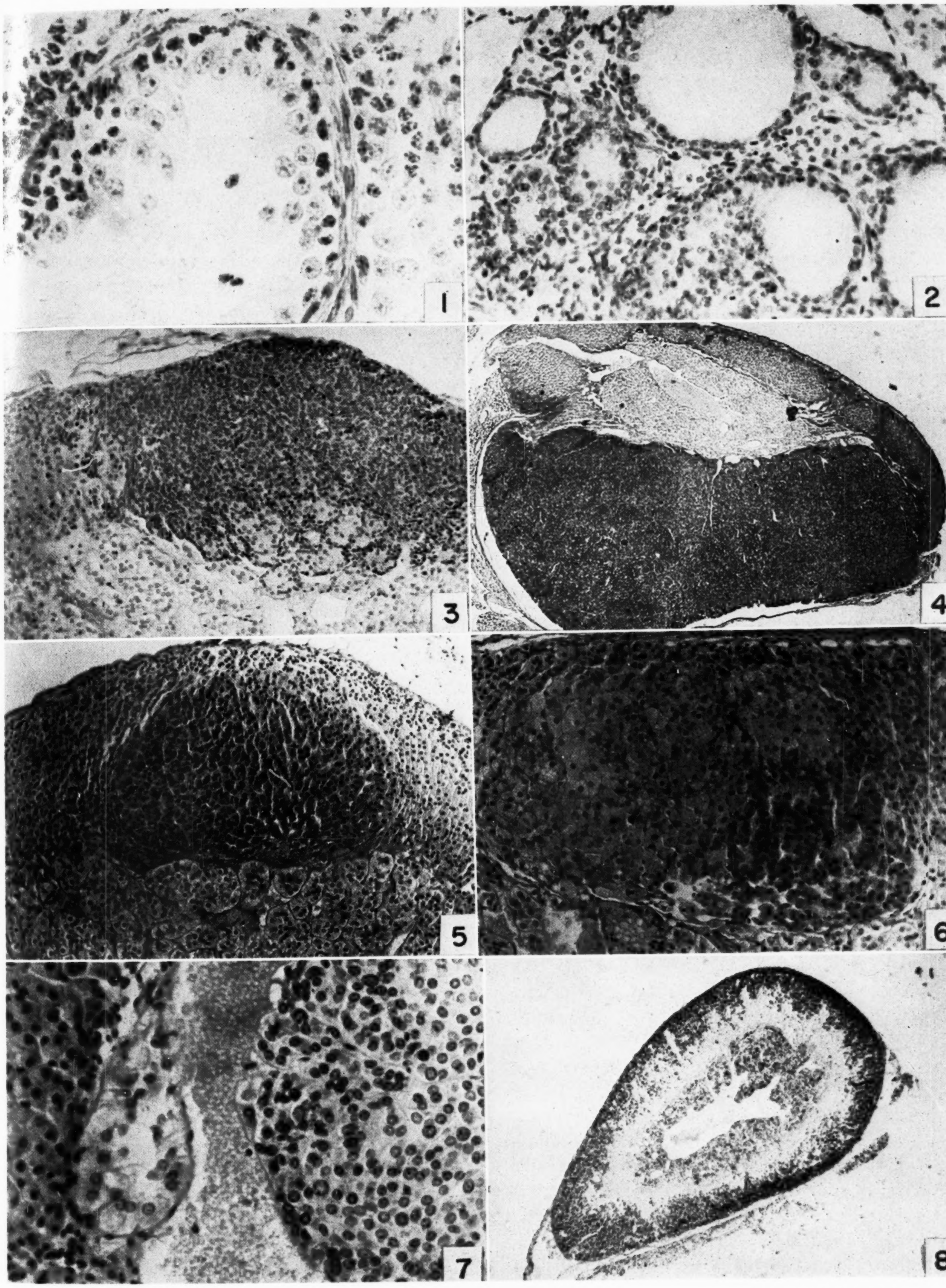
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FIG. 1.—Adrenal tumor of a 17-mo. gonadectomized DBA × CE F_1 female WK 416 showing an attempt at an arrangement similar to the pattern found in the seminiferous tubules of a cryptorchid testis. ×534.

FIG. 2.—Adrenal tumor of a 19-mo. gonadectomized DBA × CE F_1 female WK 486 with an attempt at a pattern similar to the germinal epithelium of the ovary. ×200.

FIG. 3.—Nodule of A cells with a beginning area of hyperplastic B cells at the base of the nodule. B cells are unusual in intact animals. From the adrenal of a 20-mo. intact CE × DBA F_1 male WK 774. ×100.

FIG. 4.—Adrenal of an intact 20-mo. CE × DBA F_1 female WK 782 with an extensive tumor made up of A cells. ×25.

FIG. 5.—Small typical fasciculata nodule (FN) observed in a 21-mo. CE × DBA F_1 male WK 806. ×100.

FIG. 6.—A more advanced FN in the other adrenal of the 21-mo. CE × DBA F_1 male WK 806. ×150.

FIG. 7.—Section of adrenal of a 20-mo. CE × DBA F_1 intact female WK 782 showing from left to right normal cortex, normal medullary cells, and medullary tumor. ×350.

FIG. 8.—Adrenal gland of a 26-mo. intact DBA × CE F_1 male WK 936 with extensive hyalinization and degeneration of the area between the cortex and medulla. ×30.

Growth of Carcinoma Implants in Fed and Fasted Rats*

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A number of investigations have been made recently concerning the comparison of protein metabolism in normal tissues to that in neoplastic tissues. These were reviewed by Zamecnik (9) and by Mider (3). Mider *et al.* (4) have referred to tumor tissue as a "nitrogen trap," indicating that it probably tends to remove amino acids from the body pool without permitting any appreciable return to that pool. In his review, Mider raises several questions which are important in formulating a better concept of the tumor-host relationship with regard to tumor protein metabolism. These remained unanswered, and it was suggested that they would be amenable to research with isotopically labeled precursors. Among these questions were: "How much exchange exists between the neoplasm and its host with regard to amino acids? Is the nitrogen metabolism of the cancer essentially a one-way passage? Is the necrotic material in a tumor inert, or do split-products gain access to the blood stream?"

Shemin and Rittenberg (7) studied the incorporation of glycine-N^{15} into normal rat tissues and into sarcoma R39. Although the atoms per cent excess of N^{15} at several times after the administration of glycine-N^{15} is given, the above questions are not answered, since data on the tumor weights and dilution due to rapid growth are not included. More recently, Norberg and Greenberg (5) have made a study of the incorporation of glycine-1-C^{14} into the tissues of normal and lymphosarcoma-bearing mice. This was presented as change of specific radioactivity of the proteins with time, over a period of 48 hours. The effect of fasting was studied, but total tissue content of the isotope cannot be calculated from their data. When the data of Tyner *et al.* (8) were plotted as total radioactivity versus time, the total radioactivity in the livers of fed rats that had been given glycine-2-C^{14} declined with time, whereas that of the tumors was maintained.

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With reference to the questions raised by Mider (3, 4), the tumor most likely to show a relatively small return of assimilated amino acid to the body pool would be a very malignant and rapidly growing tumor. Consequently, for the initial experiments we chose the Flexner-Jobling carcinoma, one of the most rapidly growing rat tumors available. Protein (amino acid) turnover was studied by means of isotope experiments, and fasting animals were used to limit protein turnover to a host-tumor relationship. Measurement of total tumor mass was also an important feature of the experiments.

EXPERIMENTAL

The animals used were female, albino rats obtained from the Holtzman-Rolfsmeyer Company (Sprague-Dawley strain) when they weighed 100–110 gm. They were kept in our laboratories 1 week on a Purina Fox Chow diet. Then Flexner-Jobling carcinoma implants were made subcutaneously at four sites on the abdominal area of each rat. Each batch of tumor mince was used for implants on twelve rats, and each such group was distributed equally through all experimental groups.

The first experiment was designed to determine the rate of tumor growth and tissue protein changes in fasted and fed tumor-bearing rats. Rats, each bearing four 10-day-old Flexner-Jobling carcinomas, were divided into three groups, twelve animals to a group. One group was sacrificed immediately to obtain initial tissue weights. A second group was fed Purina Fox Chow ad libitum. The third was fasted. After an additional 5 days, both of the latter groups were sacrificed and tissues weighed. At this time several of the rats of the fasted group were dying. Six of the 12 died on the morning of the fifth day, while the others were being sacrificed. Since no difference in tissue weights could be detected when these six were compared with the others, all twelve were grouped together in presenting the data. Tissues from each group were pooled into subgroups of three, and the total tissue pool in each case was broken up in a Waring Blendor with 9

volumes of water. Aliquots of the tumor and liver suspensions were treated with trichloroacetic acid to a final concentration of 5 per cent. The resulting precipitate was washed twice with 5 per cent trichloroacetic acid, twice with 95 per cent ethanol, and extracted 3 times with 3:1 alcohol-ether at 40° C. Finally, the precipitate was washed twice with ether and dried *in vacuo*. The resulting material was weighed and the weight used to compute per cent protein. The tissue weights and protein contents are presented in Table 1. The mean of results from twelve rats and the average difference from the mean have been presented in each case.

In 5 days the weight of tumor protein increased 345 per cent in the fed rats, 160 per cent in the fasted rats. The latter result is the more noteworthy when it is considered that these animals lost 31 per cent of their body weight and 39 per cent of their liver protein during the same period. It is thus apparent that the tumor was able to grow rapidly at the expense of the host tissues.

Further aliquots of the suspensions of liver and tumor were extracted and used for determinations of pentosenucleic acid (PNA) and desoxypentosenucleic acid (DNA), according to the method of Schneider (6). The results of these analyses are presented in Table 2. The liver DNA changed very little from that of the control group, but its PNA content decreased considerably with fasting. In the tumors, DNA increased approximately in proportion to the tissue weight, but the PNA content was held down by fasting. Hence the PNA/DNA ratios decreased with fasting in both tissues.

A second experiment was set up with an isotopically labeled protein precursor to provide more definitive results concerning the tumor-host tissue relationship in fed and fasted animals. The low

TABLE 1

BODY AND TISSUE WEIGHTS OF FASTED AND FED TUMOR-BEARING RATS

A group consisted of twelve female rats, each bearing four subcutaneous implants of Flexner-Jobling carcinoma. Tumors were implanted 10 days earlier.

	WEIGHTS*					
	Rats sacrificed initially		Rats fed 5 days		Rats fasted 5 days	
TISSUE	Fresh wt. (gm.)	Protein content (per cent)	Fresh wt. (gm.)	Protein content (per cent)	Fresh wt. (gm.)	Protein content (per cent)
Body	174 ±7.2		173 ± 6.0 182 ±10		172 ±6.5 118 ±1.7	
Tumor†	4.14±1.20	12.1±0.2	12.97± 1.64	17.2±0.7	7.52±2.22	17.3±0.4
Brain	1.62±0.03		1.67± 0.05		1.66±0.02	
Liver	7.00±0.15	21.2±0.1	9.17± 0.42	21.9±1.3	3.51±0.12	25.8±2.0
Kidneys	1.28±0.03		1.29± 0.03		1.00±0.06	
Thymus	0.39±0.02		0.35± 0.03		0.06±0.01	
Spleen	0.92±0.07		2.58± 0.15		0.59±0.13	
Heart	0.57±0.01		0.56± 0.02		0.39±0.01	

* The mean with average mean deviation.
† Per rat (sum of four tumors).

TABLE 2

MEASUREMENTS OF NUCLEIC ACIDS IN THE TISSUES OF FASTED AND FED TUMOR-BEARING RATS

A group consisted of twelve female rats, each bearing four subcutaneous implants of Flexner-Jobling carcinoma, made 10 days earlier.

			NUCLEIC ACID ANALYSES			
GROUP	TISSUE COMPONENTS		mg/gm fresh wt.*	mg/gm protein	mg. in the entire tissue	RATIO OF PNA/DNA
Rats sacrificed initially	Liver	PNA	7.6±0.2	36	53	3.4
		DNA	2.2±0.1	10.4	15	
	Tumor	PNA	5.5±0.1	45	23	1.2
		DNA	4.5±0.1	37	15	
Rats fed 5 days	Liver	PNA	7.4±0.2	34	68	3.2
		DNA	2.3±0.1	10.5	21	
	Tumor	PNA	3.7±0.2	22	48	0.84
		DNA	4.4±0.2	26	57	
Rats fasted 5 days	Liver	PNA	7.2±0.4	28	23	1.9
		DNA	3.6±0.2	14	12	
	Tumor	PNA	3.5±0.2	20	26	0.72
		DNA	4.8±0.0	28	36	

* The mean with average mean deviation.

variability in the first experiment indicated that smaller groups would yield significant results. Sixty female rats of the same specifications described earlier were selected into ten groups of six each, with identical average weights for each group. Six of these groups (36 rats) were inoculated subcutaneously with Flexner-Jobling carcinoma, each at four sites. After $9\frac{1}{2}$ days each of the 60 rats was injected intraperitoneally with 200 μg. of glycine-2-C^{14} (2,900,000 counts/min).[1] After a lapse of 12 hours, at which interval it was indicated in earlier data that the proteins would have reached maximum radioactivity (8), one group of six normal rats and one of six tumor-bearing rats were sacrificed and tissue weights obtained. The other groups were now put on fasting or feeding regimes as indicated. As each group was sacrificed, it was divided into two pools, each pool consisting of the tissues from three rats. Each liver, kidney, and tumor pool was dispersed in 9 volumes of water in the Waring Blendor, and protein isolated as described for the first experiment. The isolated proteins were finely ground in a mortar with 25 per cent ethanol-75 per cent water and collected on duplicate paper discs by filtration. They were dried *in vacuo*, weighed, and counted in internal flow counters. The radioactivity was corrected for self-absorption and expressed as counts/min/mg of protein. All samples were counted for a sufficiently long period to obtain a 5 per cent statistical accuracy.

The results of this experiment are presented in

[1] Obtained from Tracerlab, Inc., on allocation by the United States Atomic Energy Commission.

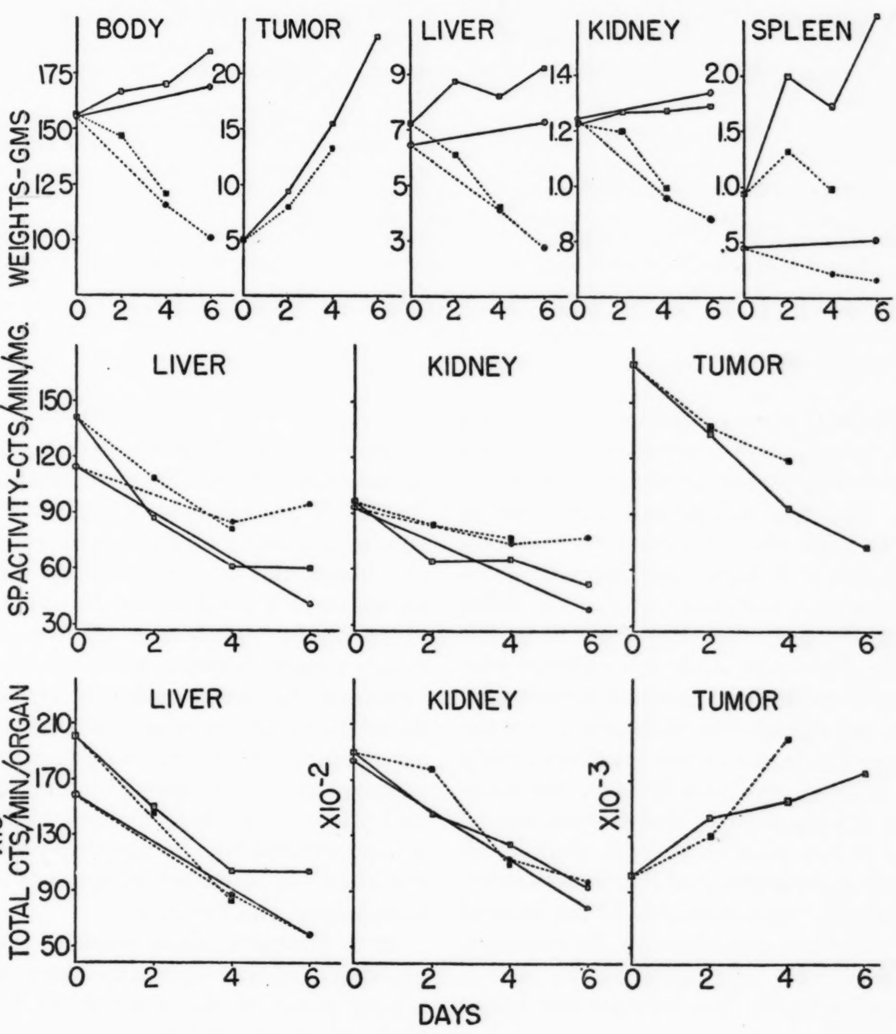

CHART 1.—○——○ fed control rats; ●------● fasted control rats; □——□ fed tumor-bearing rats; ■------■ fasted tumor-bearing rats.

Top row: changes in body and tissue weights with feeding and fasting.

Center row: changes in specific radioactivity with feeding and fasting.

Bottom row: changes in total radioactivity per organ with feeding and fasting.

Chart 1. The top row in Chart 1 gives the weight changes in body (including the tumors), tumors, liver, kidney, and spleen. In this experiment the increase in tumor weight to 4 days was rapid in both fed and fasted groups, with less difference between the two than at 5 days in the earlier experiment. McEwen and Haven (2) noted an increase in liver weight in tumor-bearing animals. With the much smaller tumors in these experiments one can still observe an increase of liver weight in the tumor-bearing group. A much more pronounced increase is notable in the spleens of the tumor-bearing groups, which even fasting does not completely suppress. In the center row of Chart 1 are expressed the changes in specific radioactivity of the liver, kidney, and tumor proteins. The individual plots of the two sub-groups were averaged, in each case, since it was confusing to have eight points in juxtaposition at some places in the charts. The two sub-groups gave closely agreeing results with the exception of one case (tumors of fed rats at 2 days), and here the average fitted into position with the trend of the other points. The specific radioactivity of all three tissues is declining. However, since the radioactivity of tumor protein declines because of dilution by further growth and the specific radioactivity of the precursor glycine declines to a low level very early after a single dose of glycine-2-C^{14} (8), the more significant plot is presented in the bottom row of Chart 1. Here it can readily be seen that in all groups the total radioactivity of liver and kidney proteins, presumably representative of the normal body proteins, declines considerably. In contrast, the total radioactivity of tumor shows a significant increase, whether the animals are fed or fasted.

Since glycine is a precursor of both proteins and nucleic acid purines and both are included in the material referred to here as "protein," it was appropriate to separate the two and to determine the relative proportions of the radioactivity present in each. Six samples, representative of different experimental times and of liver and tumor from the same animal groups, were used. Each was extracted with sodium chloride according to the method of Hurlbert and Potter (1) to separate nucleic acid from protein. After washing and drying, the proteins and nucleic acids were plated directly and radioactivity measured as before. The results are given in Table 3. The proportion of the radioactivity in the nucleic acids is sufficiently small and regular that routine extraction of the samples did not seem justified, since the interpretation of results would be unchanged. Apparently nucleic acid and protein activities are changing in a parallel manner.

TABLE 3

RADIOACTIVITY DISTRIBUTION BETWEEN PROTEIN AND NUCLEIC ACIDS OF LIVER AND TUMOR "PROTEINS"

Each figure represents duplicate counts on one of the protein samples obtained from a pool of the tissues of three rats.

		TOTAL RADIOACTIVITY/100 MG DRIED "PROTEIN"			
		Protein			PERCENTAGE
		Before	After	Nucleic	PRESENT AS
GROUP	TISSUE	extraction	extraction	acid	NUCLEIC ACID
Rats sacrificed initially	liver	14,100*	12,100*	370*	2.6
Rats fed 4 days	liver	6,100	5,900	415	6.8
Rats fasted 4 days	liver	8,400	6,500	360	4.3
Rats sacrificed initially	tumor	17,000	14,000	3,080	18.1
Rats fed 4 days	tumor	9,300	7,000	1,690	18.2
Rats fasted 4 days	tumor	12,900	14,000	1,900	14.7

* Counts/min.

DISCUSSION

It is apparent from these data that the nitrogen metabolism of the Flexner-Jobling carcinoma is largely, if not entirely, a "one-way passage." The tumor is able to obtain all its requirements from the blood and to grow rapidly when the host is fasted and forced to maintain the blood constituents by catabolism of normal tissues. The tumor protein is not available to the body for conversion to fuel even under the stress of starvation. Since the tumors at the final time studied in these experiments were necrotic, it seems likely that radioactivity, if released by the autolysis of central portions of these tumors, must be efficiently reincorporated by the growing portions to permit the observed rapid net increase of radioactivity in both fasted and fed rats.

It is probable that some of the more slowly growing tumors, especially those which have retained some of the specialized functions of the tissue of origin, will not show as clear a result and will permit greater exchange of amino acids with the body pool. The experiments described here must be repeated with tumors of various types and with normal growing tissues before it can be

concluded that uncontrolled growth is inversely correlated with the rate of return of amino acids to the body pool.

SUMMARY

Control rats and rats bearing multiple implants of Flexner-Jobling carcinoma were fed or fasted and the weight changes determined for tumors and normal tissues. Glycine-2-C^{14} was used to label the tissue proteins and to permit a study of amino acid exchange. Animals fasted 5 days lost 31 per cent of their body weight and 39 per cent of their liver protein. Tumors on such animals grew almost as rapidly as on fed rats. It was observed that in either fed or fasted rats, the specific radioactivity of the proteins and nucleic acids of normal tissues declined with time, as did the total radioactivity per organ. In contrast, the total radioactivity of the tumor proteins increased rapidly. It was concluded that protein metabolism in the Flexner-Jobling carcinoma is essentially, if not completely, a "one-way passage," and that the proteins of this tumor are not available to the host for fuel during starvation. It is suggested that the type of experiment described herein be used to survey host-tumor relationships for a variety of tumors, including some which grow more slowly and are less anaplastic than the Flexner-Jobling carcinoma.

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In Vivo Studies on Incorporation of Glycine-2-C^{14} into Proteins and Nucleic Acid Purines*

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A number of workers, using different approaches, have attacked the problem of protein and nucleic acid biosynthesis by time studies. Shemin and Rittenberg (32), using glycine-N^{15}, reported an extensive study of nitrogen metabolism in the sarcoma-bearing rat. They found that N^{15} was incorporated into tumor protein less rapidly than into liver protein, and that over a period of time its concentration decreased more slowly in tumor. Although Griffin *et al.* (16) have studied the uptake of glycine-2-C^{14} by normal tissues and by azo dye-induced hepatomas in rats, they did not separate nucleic acids from proteins nor did they present their data in the form of a turnover study. They found that maximum incorporation of glycine into the liver protein occurred within 2 hours, whereas maximum incorporation into hepatoma protein occurred at 12 hours. Using mice bearing Gardner lymphosarcoma, Norberg and Greenberg (27) also studied the incorporation of glycine into proteins. In this species, maximum incorporation of glycine into protein was observed at about 2 hours. A different type of time study was reported by Hammarsten's group (12) who investigated differences in the incorporation of glycine during the formation of proteins and polynucleotides in regenerating tissues. In their experiments, the time variable was the interval after partial hepatectomy in the rat or after the administration of phenylhydrazine to the hen. The interval after the administration of isotopic glycine was kept constant.

In the present study, glycine, a component of protein as well as a precursor of purines (6, 18, 21, 30, 35, 37), was chosen as the labeled compound to be administered. Interesting differences have been observed between experiments in which small-molecule carbon precursors of purines, such as glycine, have been used and those in which preformed purines or phosphorus have been administered. The first evidence that the purines of nucleic acids could be synthesized from small molecules was the observation of Barnes and Schoenheimer in 1943 (2) that isotopic ammonia was incorporated into the purines and pyrimidines of nucleic acids in the rat and into uric acid in the pigeon. Sonne, Buchanan, and Delluva (9, 35–37), studying uric acid synthesis in the pigeon, discovered the roles of glycine, formate, and carbon dioxide and elucidated the origin of each carbon in the purine ring. Heinrich and Wilson (18) extended this work to the rat. Many studies on the biosynthesis of polynucleotides were made employing P^{32}, reviewed by Hevesy (19), or adenine, as in the investigations of Brown and associates (7, 8, 15). Both phosphorus and adenine were found to be incorporated at an appreciable level into PNA but to a considerably lesser extent into DNA of nongrowing tissues. These findings have led to the concept of the biochemical stability of DNA in nonproliferating cells. In contrast to these results, LePage and Heidelberger (22, 23) have reported that glycine is rapidly and extensively incorporated into the purines of both DNA and PNA in tissues of the adult rat. Independent studies showing considerable incorporation of other small-molecule precursors into DNA purines have been made by Elwyn and Sprinson (13) with glycine-2-C^{14} and with serine-3-C^{14}, and by Totter *et al.* (38, 39) with isotopic formate. Furst and Brown (14) have recently confirmed the general observations outlined above by experiments in which glycine-N^{15} and adenine-8-C^{14} were administered simultaneously to rats: in normal liver the incorporation of *glycine* into the purines of PNA was only 2.5–3 times greater than into the purines of DNA, whereas in the same animals 50–60 times as much *adenine* was incorporated into PNA as into DNA. In other experiments on the incorporation of glycine into purines, Heidelberger and LePage (17) have reported that the glycine molecule is largely incorporated as such into the purines

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of both PNA and DNA, and not merely via formate by simple exchange with the ureide carbons 2 and 8: i.e., extensive synthesis of the main carbon skeleton of both PNA and DNA purines takes place *in vivo*. Thus, it has been concluded that two pathways of nucleic acid synthesis exist, one using adenine, carbohydrate, and phosphorus; the other, small-molecule precursors which may combine to form an open-chain intermediate—perhaps a derivative of 5(4)-amino-4(5)-imidazole carboxamide (26, 29, 33)—to which carbohydrate and phosphorus have already been attached. Bendich (5) has reported the isolation from mixed organs of two fractions of DNA, differing in solubility and in distribution of isotope, after the administration of labeled formate. The latter work suggests that the two metabolic pathways for nucleic acid synthesis may lead to two different types of DNA differing in stability.

In order to obtain further information concerning the interrelationships between protein and nucleic acid biosynthesis, a kinetic study was undertaken with glycine-2-C^{14} in tumor-bearing rats. The objective of this work was to compare the synthetic processes in liver and tumor at a number of different time intervals after the administration of the precursor. The following points were investigated: (*a*) relative rates of incorporation into DNA and PNA purines; (*b*) turnover half-times; (*c*) comparison of normal and neoplastic tissues. A preliminary report has been made (40).

EXPERIMENTAL

Female rats,[1] weighing 150 gm. and bearing 10-day multiple transplants of Flexner-Jobling carcinoma, were given a single dose of 0.5 mg. of glycine-2-C^{14} [2] by stomach tube. Three animals were used for each point on the time curve ($\frac{1}{2}$, 1, 2, 4, 6, 12, 24, 36, 48, and 96 hours for tumor-bearing rats, and 96, 168, and 288 hours for normal rats). Stock grain diet and tap water were provided. At the appropriate time the animals were killed by decapitation and their livers, tumors, and kidneys processed individually according to (*a*) the method of LePage and Heidelberger (23) or (*b*) the method of Hurlbert and Potter (20) to obtain protein, DNA, and PNA preparations. The tissues were homogenized in cold 0.4 N perchloric acid, the residue washed several times with this concentration of perchloric acid, twice with 95 per cent ethanol containing 0.01 N perchloric acid, and twice with 95 per cent ethanol, then extracted 3 times with 3:1 ethanol-ether at 40°. In method (*a*) this residue was dissolved in 1 N NaOH, incubated overnight at 37°, and chilled prior to precipitation of protein plus DNA by the addition of trichloroacetic acid. From this supernatant fluid PNA was isolated as the barium salt; from the residue DNA was obtained by extraction with hot 0.3 M barium perchlorate; protein remained. In method (*b*) the tissue residue after the extraction of lipids was extracted with 10 per cent NaCl, pH 7–8, at 100° for 1 hour, then re-extracted for 30 minutes. Sodium nucleates were obtained from these combined extracts. To separate PNA and DNA the sodium nucleates were dissolved in 0.1 N NaOH and incubated at 37° overnight, then chilled and acidified with HCl. The precipitated DNA was quickly separated by centrifugation; the supernatant fluid contained depolymerized PNA. Preparations of DNA and PNA, analyzed by the diphenylamine and orcinol color reactions (31), were considered of adequate purity if less than 10 per cent of the undesired nucleic acid was present.

To obtain the free purines, pooled aliquots of DNA or PNA samples (containing about 2 mg. purines) were subjected to hydrolysis (1 hour in 1 N HCl at 100° for PNA, 20 min. for DNA), followed by removal of barium ions (if present) with sulfuric acid and a partial purification on a short column (6 × 10 mm.) of Dowex-50 cation exchange resin. The diluted hydrolysates were placed on the column and washed once with water, then with 1.5 ml. of 2 N HCl, which removed the pyrimidine nucleotides and most of the dark-colored hydrolysis products. The desired purines were then eluted with 4 ml. of 6 N HCl and the almost colorless eluate evaporated to dryness in a vacuum oven at 40°. In order to remove any glycine which might have been carried along as contaminant, this residue was routinely treated with 1 mg. ninhydrin in aqueous solution, heated 10 min. at 100°, and again evaporated to dryness to expel formaldehyde derived from the methylene carbon of glycine. In preliminary experiments this precaution lowered the values for specific activity of adenine and guanine by less than 5 per cent, but was nevertheless considered desirable, since in the ensuing chromatographic separation glycine was found to move with adenine. Adenine and guanine were separated by paper chromatography using the solvent system of Smith and Markham (34), *tert*-butanol-HCl-water. One of the solvent systems employed by Carter (10), isoamyl alcohol-5 per cent aqueous Na_2HPO_4, was used in a few experiments. It was discarded because, owing to the presence of salt in the paper and the immobility of

[1] Obtained from the Holtzman-Rolfsmeyer Rat Co., Madison, Wis.

[2] Specific activity 15,000,000 counts/min/mg; obtained from Tracerlab, Inc., on allocation by the U.S. Atomic Energy Commission.

guanine in this system (resulting in nonuniform distribution throughout the thickness of the paper), adequate corrections for self-absorption in the measurements of radioactivity could not be made. The purine-containing areas of the chromatogram were visible under ultraviolet illumination from a Mineralight lamp (10); discs of uniform size were cut from these and counted directly in internal flow counters to within 10 per cent statistical error and were corrected for self-absorption.[3]

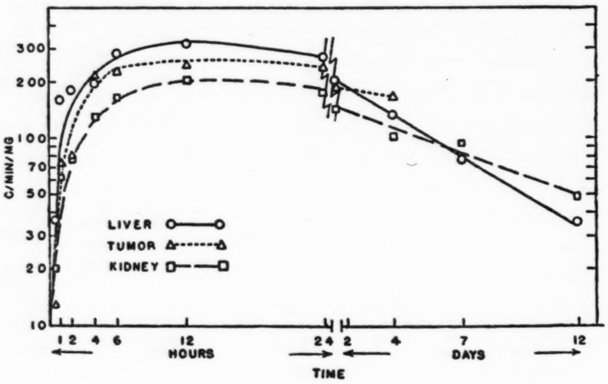

CHART 1.—Protein; specific activity (logarithmic ordinate) versus time.

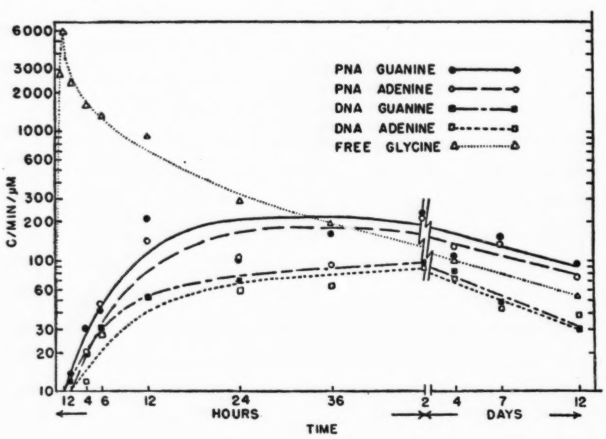

CHART 2.—Liver; DNA and PNA purines and free glycine. Specific activity (logarithmic ordinate) versus time.

After having been counted, the purines were eluted (41) from the filter paper discs and analyzed in a Beckman model DU spectrophotometer. Measurements of absorption were made at 260 mμ; the position of the characteristic absorption maximum was checked as a criterion of purity. Appropriate blanks were used to correct for ultraviolet absorption by the paper. The values reported for specific activities are averages of analyses on at least three such paper discs.

[3] The authors are indebted to Mrs. Edith Wallestad, Miss Nancy Lake, Miss Marjorie Gilbert, and Mr. A. G. Malman for their assistance in the measurements of radioactivity.

Samples of proteins obtained as described above were subjected to wet combustion (Van Slyke-Folch) and counted as $BaCO_3$. The values reported for specific activity of proteins are averages of values obtained from the tissues of individual animals.

In addition, the specific activity of free glycine in each tissue was determined (4). Perchloric acid was removed from the pooled extracts obtained after homogenization of the tissues. Formaldehyde, liberated from glycine by ninhydrin, was distilled from the mixture and an aliquot analyzed colorimetrically with chromotropic acid; the dimedon precipitate (carrier added) was made and counted directly. The amount of free formaldehyde in the tissues was found to be negligible, compared to that of free glycine.

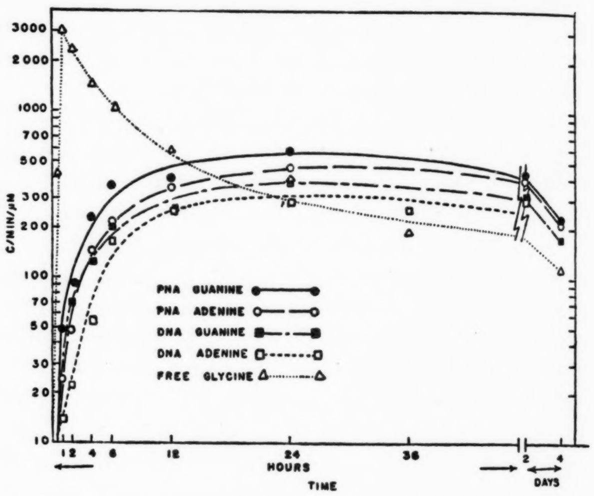

CHART 3.—Flexner-Jobling carcinoma; DNA and PNA purines and free glycine. Specific activity (logarithmic ordinate) versus time.

Half-times were determined directly from the descending portions of the curves in Charts 1 and 2.

RESULTS AND DISCUSSION

The results are presented in the form of semilogarithmic plots of the specific activity-time relationships, with Chart 1 giving the data for proteins of liver, kidney, and tumor; Chart 2, for DNA and PNA purines and free glycine of liver; Chart 3, for DNA and PNA purines and free glycine of tumor. From these graphs experimental half-times have been determined and are presented in Table 1. It was not possible to apply the theoretical considerations of Zilversmit *et al.* (45, 46) because (*a*) free glycine cannot be the *immediate* precursor of nucleic acid purines (see discussion later) and (*b*) pool size of purines was not determined; hence, calculations for true rates of turn-

over were not made. However, a time study of this type may be considered a more valid approach to the investigation of "turnover" than a study in which incorporation of a precursor is observed at only a single time interval which gives misleading results. It should be pointed out that single doses of relatively minute amounts of glycine containing radiocarbon were administered in these tracer experiments, so that the body pool of glycine was not flooded with unphysiological amounts of this compound, which is an unfortunate but frequent consequence of the use of stable isotopes. In these experiments no tumor-bearing animals were studied beyond 96 hours (i.e., 14 days after the animals received multiple subcutaneous implants of Flexner-Jobling carcinoma) in order to minimize misleading data based on necrotic tumors.

In the case of the proteins (Chart 1), the isotope was incorporated most rapidly into the proteins of the liver and the highest peak of specific activity was reached in that tissue. However, the specific activity of the free glycine during the first 2 hours (Charts 2 and 3) was considerably higher in liver than in the other tissues, as would be expected in an experiment in which the glycine was administered orally to the animals. In each tissue studied, the maximum protein specific activity was observed at about 12 hours. This is not in accord with the data of Griffin *et al.* (16), who reported peak incorporation of glycine into protein of liver at 2 hours and into protein of hepatoma at 12 hours. However, their observation of this 2-hour peak in liver rests upon a single point not in agreement with the trend of their curves, and no information is given as to the number of animals involved in this particular experiment. In view of the high specific activity of free glycine in the liver at that early time, it is possible that their protein samples were not completely free of this contaminant. If this 2-hour point is disregarded, their data do not conflict with our observations. The data of Norberg and Greenberg (27) for a different species, the mouse, indicated an earlier peak of specific activity in all tissues studied; this is not surprising in view of the known differences in metabolic rates between mice and rats. Recently a review of studies on protein synthesis has been presented by Zamecnik (42). In contrast to studies *in vivo*, such as Griffin's and our present report, in which incorporation of the label was shown to be faster and greater in liver than in tumor, slice technics have demonstrated the opposite (43). This has been explained (16, 44) on the basis of availability of amino acids: the tumor *in vivo* was limited by a relatively poor and mainly peripheral circulation, whereas *in vitro* tumor slices were exposed to an environment even richer in amino acids than blood and were able to surpass liver slices in protein synthetic ability under these circumstances.

One of the assumptions in Zilversmit's theoretical development of the concept of turnover is that the tissue under consideration is in the steady state for the compound studied, i.e., rate of synthesis equals rate of degradation. In tumor this is not true. Anabolism predominates over catabolism, and the descending portions of the specific activity-time curves (Charts 1 and 3) represent not so much a *turnover* (degradation, exchange, and resynthesis) of protein or nucleic acids as a *dilution* by protein or nucleic acids newly synthesized from less radioactive precursors. For this reason we do not wish to present our data on tumors in the form of turnover half-times, which in this case would be misleading; in Table 1 figures are presented only for liver. Mider (25) has suggested that tumor may be regarded as a "nitrogen trap," i.e., that protein once formed in tumor tissue may not thereafter be available for the normal metabolic processes of the animal. An approach to the investigation of this concept is the comparison of a normal tissue to tumor on the basis of total amount of radioactivity incorporated and the changes occurring over a period of time. When the data of Chart 1 are replotted, total counts per minute per organ versus time, the proteins of liver and kidney exhibit a decrease in total activity, reflecting their dynamic equilibrium with other proteins, whereas the total activity in tumor proteins apparently does not decrease, indicating almost completely anabolic nitrogen metabolism. Experiments on fed and fasted tumor-bearing rats were carried out to test this concept more rigorously. In the highly malignant Flexner-Jobling carcinoma, protein metabolism is essentially a "one-way passage," i.e., tumor proteins are not available to the host even under stress of starvation (see accompanying paper [24]).

TABLE 1

EXPERIMENTAL HALF-TIMES

Fraction	Half-time (hr.)
Protein:	
Liver	100
Kidney	170
PNA:	
Liver Guanine	220
Liver Adenine	230
DNA:	
Liver Guanine	150
Liver Adenine	160
Free Glycine:	
Component *A*	9
Component *B*	200

Specific activity-time relationships for free glycine of liver and tumor are plotted in Charts 2 and 3, along with values for nucleic acid purines. After the rapid initial uptake of radioactivity, greatest in liver, the glycine curves fall off along essentially the same line in all three tissues; the curve for kidney glycine (not plotted) is very close to that for tumor glycine. These determinations of free glycine provide a basis for comparison of the tissues with respect to compounds derived from glycine. In Table 1 half-times are presented for two arbitrarily selected regions of this common curve. Component *A* represents the slope of the glycine curve between 4 and 24 hours. During this period the isotopic glycine is incorporated rapidly into proteins and nucleic acids and is converted into serine, formaldehyde, and carbon dioxide. Component *B* represents the less steep slope of the curve between 4 and 12 days and appears to reflect the dilution resulting from the equilibrium between free glycine and tissue proteins after the isotope has reached its maximum distribution. It should be noted that specific activity of free glycine falls below that for nucleic acid purines before the end of the experiment. This is the expected relationship between a precursor and its product.

Turning now to a consideration of the nucleic acid purines, one notices that unlike the case of protein, the isotope is incorporated more rapidly and reaches a higher peak in purines of both DNA and PNA of tumor than in the corresponding components of liver, even though the specific activity of free glycine was for a short time higher in liver than in tumor. This means that incorporation of glycine into proteins is not inextricably linked with incorporation of glycine into nucleic acid purines and suggests that protein synthesis is not necessarily synchronized with the synthesis of nucleic acids.

Radioactivity was first detectable in purines in both liver and tumor DNA and PNA 1 hour after the administration of glycine (e.g., liver PNA guanine at $\frac{1}{2}$ hour: 0 counts/min/μM; at 1 hour: 7 counts/min/μM). In liver these values are not discernible on the chart. On the other hand, in *proteins* of the three tissues appreciable amounts of radioactivity (e.g., liver protein at $\frac{1}{2}$ hour: 37 counts/min/mg; at 1 hour: 160 counts/min/mg) were detectable even by $\frac{1}{2}$ hour, the shortest interval studied, in spite of the delay involved for absorption of the labeled glycine from the gastrointestinal tract, transportation to the tissues, and uptake by the individual cells. Thus, a distinct lag occurred between the time the labeled glycine was known to be within the cell (as shown by its incorporation into cellular proteins) and the time radioactivity was first detectable in purines isolated from cellular nucleic acids. This would seem to indicate that formation of an intermediate purine precursor more complex than glycine is necessary. These data do not support the hypothesis that a metabolite necessary for nucleic acid synthesis in the tumor is provided by the liver. Preliminary *in vitro* studies[4] indicate that tumor cells are able to carry out the entire process of incorporation of glycine into nucleic acid purines.

The similarity between the curves for DNA and PNA should be pointed out. The present study is a further substantiation of the extensive synthesis of DNA from small-molecule precursors even in nongrowing tissues such as adult liver (23). In each case, incorporation of glycine into guanine was somewhat greater than into adenine. Since more guanine than adenine is present in PNA (11) of mammalian tissues, considerably more guanine than adenine must be synthesized from glycine in order to result in this relationship. Similar observations have been made by other investigators using small-molecule precursors of nucleic acids (1, 2, 6, 12, 18).

Chart 2 shows that for both PNA and DNA of liver, the curves for guanine and adenine are strikingly parallel, resulting in nearly identical half-times (Table 1) for the purine components of each nucleic acid. A similar parallel relationship exists between the purines of tumor nucleic acids. In spite of the fact that amounts of incorporation must differ (see above), it appears that our turnover rates are essentially the same for purines in a particular nucleic acid. Thus, at least the two purines of nucleic acids must be degraded and resynthesized together in an organized fashion.

The rates at which both phosphorus (3) and orotic acid (20, 28) are incorporated into nuclear PNA differ from their rates of incorporation into cytoplasmic PNA, and, therefore, attention should be drawn to the fact that PNA in the present study was a mixture of both types. Experiments are now in progress employing cell fractionations to study two precursors, P^{32} and glycine-2-C^{14}, in the same animal. This should shed further light on the problem of intracellular relationships of PNA and DNA synthesis.

SUMMARY

A kinetic study was made of the incorporation of glycine-2-C^{14} into proteins and nucleic acid purines of rat liver and tumor. From the subsequent breakdown of labeled moieties half-times were calculated. Determinations of specific activity of free glycine in the tissues were also carried out.

[4] G. A. LePage, unpublished data.

Isotopic glycine was incorporated into proteins less than ½ hour after it was administered by stomach tube. In proteins of liver, Flexner-Jobling carcinoma, and kidney the peaks of specific activity occurred at about 12 hours.

The free purines were isolated from hydrolysates of PNA and DNA by paper chromatography. Radioactivity was first detectable in nucleic acid purines about 1 hour after the administration of glycine. In PNA and DNA purines from liver and tumor, peaks of specific activity occurred at about 24 hours; specific activity of DNA purines was somewhat lower than that of corresponding PNA purines. Nearly identical half-times were found for adenine and guanine in each nucleic acid fraction. Higher specific activities were reached in tumor for purines, in liver in the case of the proteins.

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